

SCHOOL OF CIVIL ENGINEERING



JOINT HIGHWAY RESEARCH PROJECT

JHRP-76-6

FACTORS INFLUENCING NITROGEN
FIXATION BY SEVERAL WOODY
SHRUBS AND TREES

Robert E. McNiel



PURDUE UNIVERSITY
INDIANA STATE HIGHWAY COMMISSION



Digitized by the Internet Archive
in 2011 with funding from
LYRASIS members and Sloan Foundation; Indiana Department of Transportation

Interim Report

FACTORS INFLUENCING NITROGEN FIXATION BY SEVERAL WOODY SHRUBS AND TREES

TO: J. F. McLaughlin, Director
Joint Highway Research Project

January 13, 1976

File: 9-5-7

FROM: H. L. Michael, Associate Director
Joint Highway Research Project

Project: C-36-486

Attached is an Interim Report on the HPR Part II Research Study titled "Development of Techniques for Plant Establishment on the Roadside". The Report is of the nitrogen fixation phase of the research and is titled "Factors Influencing Nitrogen Fixation by Several Woody Shrubs and Trees". It has been authored by Mr. Robert E. McNiel, a graduate instructor in research in the Department of Horticulture, School of Agriculture under the direction of Professor Philip L. Carpenter.

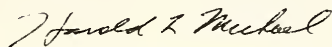
Nitrogen fixation is the process whereby the gaseous nitrogen in the atmosphere, which is useless to plants as a nutrient, is converted to a form which they can use. One type of nitrogen fixation occurs in a symbiotic relationship between a micro organism and a woody plant. Not all species of plants appear to be capable of this type of relationship which enables the plant to have use of this supplemental source of nitrogen.

Along Indiana's highways, nitrogen is the most limiting nutrient in the soils. Therefore, the use of nitrogen fixing plants which are capable of converting atmospheric nitrogen into a usable form, appears to be a potential solution to at least this one aspect of the problem. In addition, many nitrogen fixing plants are well adapted to dry, sandy conditions.

Once it has been established that a given species is capable of having a symbiotic relationship with the micro organism which enables the plant to use this supplemental source of nitrogen, many other factors become relevant. Even a species capable of fixing nitrogen will not necessarily produce the root nodules required for the process, if the environmental conditions are not conducive. The research was an investigation into some of the environmental factors that influence a plant's ability to fix nitrogen.

The Report is submitted for acceptance as partial fulfillment of the objectives of the research. It will also be forwarded to ISHC and FHWA for review, comment and similar acceptance.

Respectfully submitted,



Harold L. Michael
Associate Director

HLM:ms

cc:	W. L. Dolch	M. L. Hayes	C. F. Scholer
	R. L. Eskew	G. A. Leonards	M. B. Scott
	G. D. Gibson	C. W. Lovell	K. C. Sinha
	W. H. Goetz	R. F. Marsh	L. E. Wood
	M. J. Gutzwiller	R. D. Miles	E. J. Yoder
	G. K. Hallock	P. L. Owens	S. R. Yoder
	D. E. Hancher	G. T. Satterly	

1. Report No.	2. Government Accession No.	3. Recipient's Catalog No.	
4. Title and Subtitle FACTORS INFLUENCING NITROGEN FIXATION BY SEVERAL WOODY SHRUBS AND TREES		5. Report Date January 1976	6. Performing Organization Code C-36-48G
7. Author(s) Robert E. McNiel		8. Performing Organization Report No. JHRP-76-6	
9. Performing Organization Name and Address Joint Highway Research Project Civil Engineering Building Purdue University W. Lafayette, Indiana 47907		10. Work Unit No.	11. Contract or Grant No. HPR-1(12) Part II
12. Sponsoring Agency Name and Address Indiana State Highway Commission State Office Building 100 North Senate Avenue Indianapolis, Indiana 46204		13. Type of Report and Period Covered Interim Report	
		14. Sponsoring Agency Code CA 387	
15. Supplementary Notes Conducted in cooperation with U.S. Department of Transportation, Federal Highway Administration. Research Study titled "Development of Techniques for Plant Establishment on the Roadside".			
16. Abstract The influences of temperature, light intensity, solar radiation and moisture on nitrogen fixation that occurs in root nodules of several species of trees and shrubs were investigated. Excised nodules of nine plant species have been characterized as to the effect of temperature on their ability to reduce acetylene (C_2H_2) to ethylene (C_2H_4). Nodules from the nonlegumes, with the exception of <u>H. rhamnoides</u> , have a maximum C_2H_2 -reduction rate at 30 C. A maximum C_2H_2 -reduction rate for nodules from <u>H. rhamnoides</u> and the legume, <u>R. pseudoacacia</u> , occurs at 20 C. At temperatures near freezing the C_2H_2 -reducing rate is very low. Reducing activity decreased sharply between 30 C and 40 C for all species except <u>E. angustifolia</u> . When <u>A. glutinosa</u> and <u>E. umbellata</u> were grown in growth chambers under 950 and 4200 ft-candle light intensities, there was no increase in C_2H_2 -reducing ability by nodules from plants under the higher light intensity versus plants grown under the lower light intensity. Field grown <u>A. glutinosa</u> did indicate response in C_2H_2 -reducing ability due to fluctuation in solar radiation or temperature or both when the plants were not under moisture stress. Ample soil moisture was needed for nodules of <u>A. glutinosa</u> to be responsive to solar radiation or temperature. Nodules from container grown <u>E. umbellata</u> did not respond with increases in C_2H_2 reduction when solar radiation or temperature increased. The lack of response is in part believed to be due to moisture stress.			
17. Key Words Nitrogen fixation, Roadside plant establishment, Woody trees, Woody shrubs, Environmental factors affecting nitrogen fixation.		18. Distribution Statement	
19. Security Classif. (of this report) Unclassified	20. Security Classif. (of this page) Unclassified	21. No. of Pages 114	22. Price

Interim Report

FACTORS INFLUENCING NITROGEN FIXATION BY
SEVERAL WOODY SHRUBS AND TREES

by

Robert E. McNiel
Graduate Instructor in Research
Department of Horticulture
School of Agriculture

Joint Highway Research Project

File No.: 9-5-7

Project No.: C-36-48G

Prepared as Part of an Investigation

Conducted by

Joint Highway Research Project
Engineering Experiment Station
Purdue University

in cooperation with the

Indiana State Highway Commission

and the

U.S. Department of Transportation
Federal Highway Administration

The contents of this report reflect the views of the author who is responsible for the facts and the accuracy of the data presented herein. The contents do not necessarily reflect the official views or policies of the Federal Highway Administration. This report does not constitute a standard, specification, or regulation.

Purdue University
West Lafayette, Indiana
January 13, 1976

FACTORS INFLUENCING NITROGEN FIXATION BY SEVERAL WOODY SHRUBS AND TREES

Highlight Summary

Nitrogen fixation is the process whereby the gaseous nitrogen in the atmosphere, which is useless to plants as a nutrient, is converted to a form which they can use. One type of nitrogen fixation occurs in a symbiotic relationship between a micro organism and a woody plant. Not all species of plants appear to be capable of this type of relationship which enables the plant to have use of this supplemental source of nitrogen.

Along Indiana's highways, nitrogen is the most limiting nutrient in the soils. Therefore, the use of nitrogen fixing plants which are capable of converting atmospheric nitrogen into a usable form, appears to be a potential solution to at least this one aspect of the problem. In addition, many nitrogen fixing plants are well adapted to dry, sandy conditions.

Once it has been established that a given species is capable of having a symbiotic relationship with the micro organism which enables the plant to use this supplemental source of nitrogen, many other factors become relevant. Even a species capable of fixing nitrogen will not necessarily produce the root nodules required for the process, if the environmental conditions are not conducive. The research was an investigation into some of the environmental factors that influence a plant's ability to fix nitrogen.

The influences of temperature, light intensity, solar radiation and moisture on nitrogen fixation that occurs in root nodules of several species of trees and shrubs were investigated.

Excised nodules of nine plant species [Alnus glutinosa (L.), Gaertn (European Alder), A. rugosa (DuRoi) Spreng. (Speckled Alder), Comptonia peregrina (L.) Coult. (Sweet fern), Elaeagnus angustifolia L. (Russian olive), E. commutata Bernh. (Silver berry), E. umbellata Thunb. (Autumn olive), Hippophae rhamnoides L. (Sea Buckthorn), Robinia pseudoacacia L. (Black Locust) and Shepherdia argentea Nutt. (Buffalo berry)] have been characterized as to the effect of temperature on their ability to reduce acetylene (C_2H_2) to ethylene (C_2H_4). Acetylene reduction has been shown by others to be an effective means of determining N_2 fixation by root nodules. Nodules from the nonlegumes, with the exception of H. rhamnoides, have a maximum C_2H_2 -reduction rate at 30 C. A maximum C_2H_2 -reduction rate for nodules from H. rhamnoides and the legume, R. pseudoacacia, occurs at 20 C. At temperatures near freezing the C_2H_2 -reducing rate is very low. The rate increases rapidly as the temperature increases until the maximum rate is obtained. Reducing activity decreased sharply between 30 C and 40 C for all species except E. angustifolia.

When A. glutinosa and E. umbellata were grown in growth chambers under 950 and 4200 ft-candle light intensities, there was no increase in C_2H_2 -reducing ability by nodules

from plants under the higher light intensity versus plants grown under the lower light intensity.

Field grown A. glutinosa did indicate response in C_2H_2 -reducing ability due to fluctuation in solar radiation or temperature or both when the plants were not under moisture stress. When solar radiation and temperature increased during the day, the rate of C_2H_2 reduced increased. Ample soil moisture was needed for nodules of A. glutinosa to be responsive to solar radiation or temperature.

Nodules from container grown E. umbellata did not respond with increases in C_2H_2 reduction when solar radiation or temperature increased. The lack of response is in part believed to be due to moisture stress. When solar radiation, temperature and evaporation were high, the C_2H_2 -reducing ability of the nodules decreased as the day progressed.

When disinfecting nodules for use on culture media, the type of disinfectant used influenced the rate of C_2H_2 -reducing activity by nodules from A. glutinosa. A 1% $AgNO_3$ treatment for 1 or 10 min caused the greatest reduction in reducing activity by the nodule.

ACKNOWLEDGMENTS

The author wishes to express his sincere appreciation to his major professor, Dr. P.L. Carpenter, for his encouragement and guidance during this investigation. My appreciation is extended to Drs. W.R. Chaney, C.A. Mitchell and D.W. Nelson for serving as the other members on the advisory committee and to Drs. P.L. Carpenter, W.R. Chaney, C.A. Mitchell and L.E. Sommers for serving on the final examining committee.

The author wishes to extend appreciation to M.L. Conder, V. Peters and G. Power Hillis for their long and late hours of technical assistance and to fellow graduate students D.F. Hamilton and D.M. Maronek for their help and suggestions.

Thanks to Mrs. Ginney Cunningham for the typing of the final manuscript.

To my wife, Patricia K., I extend my thanks for her understanding and patience.

Appreciation is also expressed to the Joint Highway Research Project, the Indiana State Highway Commission and the Federal Highway Administration whose financial support for the research made it possible.

TABLE OF CONTENTS

	Page
LIST OF TABLES.....	vi
LIST OF FIGURES.....	viii
INTRODUCTION.....	1
CHAPTER I. LITERATURE REVIEW.....	3
Identification of Plants and Microorganisms.....	3
Plant Species Forming Root Nodules.....	3
Endophyte Inducing Nodulation.....	3
Nodule Formation.....	5
N ₂ Fixation.....	5
Biochemical Process.....	5
Plants Investigated.....	7
Acetylene Reduction.....	9
Development.....	9
The C ₂ H ₂ /N ₂ Conversion Factor.....	10
Replacement of N ₂ in the C ₂ H ₂ Assay.....	10
CHAPTER II. EFFECT OF TEMPERATURE ON C ₂ H ₂ REDUCING ACTIVITY ASSOCIATED WITH ROOT NODULES ON WOODY TREES AND SHRUBS.....	12
Introduction.....	12
Materials and Methods.....	14
Experiments.....	14
Plant Material.....	15
Nodule Preparation.....	15
Temperature Treatments.....	16
Measurement of Acetylene Reduction.....	18
Results and Discussion.....	19
Experiment 1.....	19
Experiment 2.....	21
Experiment 3.....	21
Experiment 4.....	25
Conclusions.....	55

CHAPTER III. FACTORS INFLUENCING THE REDUCTION OF ACETYLENE BY EXCISED ROOT NODULES FROM <u>ELAEAGNUS UMBELLATA</u> 'CARDINAL'.....	57
Introduction.....	57
Materials and Methods.....	58
Plant Material.....	58
Nodule Preparation.....	59
Effect of C_2H_2 Concentration.....	60
Effect of Incubation Temperature.....	61
Effect of Light Intensity.....	61
Diurnal Variation in C_2H_2 Reduction.....	61
Results and Discussion.....	62
Nodule Size.....	62
Effect of C_2H_2 Concentration.....	62
Effect of Temperature.....	66
Effect of Light Intensity.....	70
Diurnal Variation in C_2H_2 Reduction.....	70
Conclusions.....	80
CHAPTER IV. THE EFFECT OF TEMPERATURE, SOLAR RADIATION, AND MOISTURE ON THE REDUCTION OF ACETYLENE BY EXCISED ROOT NODULES FROM <u>ALNUS</u> <u>GLUTINOSA</u>	81
Introduction.....	81
Materials and Methods.....	83
Plant Material.....	83
Nodule Preparation.....	83
Measurement of Acetylene Reduction.....	84
Effect of Light Intensity.....	84
Diurnal Fluctuation in the Rate of Acetylene Reduction.....	84
The Day by Day Variation in the Rate of Acetylene Reduction.....	85
Results and Discussion.....	85
Effect of Light Intensity.....	85
Diurnal Fluctuation in the Rate of Acetylene Reduction.....	87
The Day by Day Variation in the Rate of Acetylene Reduction.....	95
Conclusions.....	97

	Page
CHAPTER V. THE EFFECT OF SURFACE DISINFECTANTS ON C_2H_2 REDUCTION BY NODULES REMOVED FROM <u>ALNUS GLUTINOSA</u>	98
Introduction.....	98
Materials and Methods.....	98
Results and Discussion.....	98
CHAPTER VI. C_2H_2 GAS STANDARDS.....	101
Introduction.....	101
Materials and Methods.....	101
Results and Discussion.....	102
LITERATURE CITED.....	106
APPENDIX.....	114

LIST OF TABLES

Table	Page
I-1.. The ratio acetylene reduced to nitrogen fixed..	11
II-1. The effect of temperature on acetylene reduction by nodules from <u>E. umbellata</u> when exposing the root zone with nodules to each temperature.	20
II-2. Comparison in C_2H_2 -reducing rate at 2-4 C between exposing the root zone plus nodules and exposing only excised nodules of <u>E. umbellata</u> to the temperature.....	22
II-3. The effect of temperature on acetylene reduction when using excised nodules from <u>Alnus glutinosa</u>	23
II-4. The effect of temperature on acetylene reduction when using excised nodules from <u>R. pseudoacacia</u>	24
II-5. Temperature coefficients (Q_{10}) for C_2H_2 reduction to C_2H_4 for nodules from <u>Alnus glutinosa</u>	26
II-6. Temperature coefficients (Q_{10}) for C_2H_2 reduction to C_2H_4 for nodules from <u>Alnus rugosa</u>	27
II-7. Temperature coefficients (Q_{10}) for C_2H_2 reduction to C_2H_4 for nodules from <u>Comptonia peregrina</u>	28
II-8. Temperature coefficients (Q_{10}) for C_2H_2 reduction to C_2H_4 for nodules from <u>Elaeagnus angustifolia</u>	29
II-9. Temperature coefficients (Q_{10}) for C_2H_2 reduction to C_2H_4 for nodules from <u>Elaeagnus commutata</u>	30
II-10. Temperature coefficients (Q_{10}) for C_2H_2 reduction to C_2H_4 for nodules from <u>Elaeagnus umbellata</u>	31

Table	Page
II-11. Temperature coefficients (Q_{10}) for C_2H_2 reduction to C_2H_4 nodules from <u>Hippophae rhamnoides</u>	32
II-12. Temperature coefficients (Q_{10}) for C_2H_2 reduction to C_2H_4 for nodules from <u>Robinia pseudoacacia</u>	33
II-13. Temperature coefficients (Q_{10}) for C_2H_2 reduction to C_2H_4 for nodules from <u>Shepherdia argentea</u>	34
II-14. Approximate temperature where a change in activation energy occurs with C_2H_2 reduction to C_2H_4 by nodules from seven plant species.....	47
II-15. The effect of temperature on C_2H_2 reduction to C_2H_4 in order to determine if it is reversible.....	56
III-1. The effect of whole or divided nodules on sample variation.....	63
V-1. A comparison of C_2H_4 production by excised nodules treated with various surface disinfectants for 1 or 10 minutes.....	99
VI-1. The determination of an unknown concentration of C_2H_4 from a known standard of 0.913 ppm C_2H_4	103

LIST OF FIGURES

Figure		Page
I-1.	A diagram of the pathways that may be involved in nitrogen fixation.....	6
II-1.	The effect of various incubation temperatures on C_2H_4 production by nodules from <u>Alnus glutinosa</u>	37
II-2.	The effect of various incubation temperatures on C_2H_4 production by nodules from <u>Alnus rugosa</u>	38
II-3.	The effect of various incubation temperatures on C_2H_4 production by nodules from <u>Comptonia peregrina</u>	40
II-4.	The effect of various incubation temperatures on C_2H_4 production by nodules from <u>Elaeagnus commutata</u>	42
II-5.	The effect of various incubation temperatures on C_2H_4 production by nodules from <u>Elaeagnus umbellata</u>	44
II-6.	The effect of various incubation temperatures on C_2H_4 production by nodules from <u>Shepherdia argentea</u>	46
II-7.	The effect of various incubation temperatures on C_2H_4 production by nodules from <u>Hippophae rhamnoides</u>	49
II-8.	The effect of various incubation temperatures on C_2H_4 production by nodules from <u>Robinia pseudoacacia</u>	51
II-9.	The effect of various incubation temperatures on C_2H_4 production by nodules from <u>Elaeagnus angustifolia</u>	54

Figure	Page
III-1. The effect of C_2H_2 concentration on C_2H_2 reduction to C_2H_4 by nodules from <u>E. umbellata</u> 'Cardinal' when using a 17 ml analysis flask.....	65
III-2. The effect of C_2H_2 concentration on C_2H_2 reduction to C_2H_4 by nodules from <u>E. umbellata</u> "Cardinal" when using a 265 ml analysis flask.....	67
III-3. Effect of temperature on the reduction of C_2H_4 using excised lobes of nodules from <u>E. umbellata</u> 'Cardinal'.....	69
III-4. The effect of 2 light intensities (950 and 4200 ft-candles) on the rate of C_2H_2 reduction to C_2H_4 by excised nodules from <u>E. umbellata</u> 'Cardinal'.....	71
III-5. A comparison of the diurnal variation in the reduction of C_2H_2 to C_2H_4 by excised nodules collected from <u>E. umbellata</u> 'Cardinal' on 20 June 1974 to solar radiation and air temperature.....	73
III-6. A comparison of the diurnal variation in the reduction of C_2H_2 to C_2H_4 by excised nodules collected from <u>E. umbellata</u> 'Cardinal' on 26 June 1974 to solar radiation and air temperature.....	75
III-7. A comparison of the diurnal variation in the reduction of C_2H_2 to C_2H_4 by excised nodules collected from <u>E. umbellata</u> 'Cardinal' on 16 July 1974 to solar radiation and air temperature.....	77
IV-1. The effect of 2 light intensities (950 and 4200 ft-candles) on the rate of C_2H_2 reduction to C_2H_4 by excised nodules from <u>A. glutinosa</u>	86
IV-2. A comparison of the diurnal variation in the reduction of C_2H_4 to C_2H_4 by excised nodules collected from <u>A. glutinosa</u> on 20 June 1974 to solar radiation and air temperature.....	89

Figure	Page
IV-3. A comparison of the diurnal variation in the reduction of C_2H_2 to C_2H_4 by excised nodules collected from <u>A. glutinosa</u> on 26 June 1974 to solar radiation and air temperature.....	92
IV-4. A comparison of the diurnal variation in the reduction of C_2H_2 to C_2H_4 by excised nodules collected from <u>A. glutinosa</u> on 16 July 1974 to solar radiation and air temperature.....	94
IV-5. The influence of moisture on C_2H_2 reduction to C_2H_4 by nodules obtained from <u>Alnus glutinosa</u> over a 41 day period.....	96
VI-1. A standard curve prepared for ethylene (C_2H_4) using a Varian 1200 gas chromatograph.....	105

INTRODUCTION

Nitrogen content in plants is exceeded only by the quantities of hydrogen, carbon and oxygen. With plants and animals nitrogen is an important part of proteins and nucleic acids. Unlike the other three elements mentioned above, nitrogen is not combined with any other element in its most plentiful form--atmospheric nitrogen (N_2).

Before nitrogen can be used by a plant it has to be in a combined form such as nitrate or ammonium. However, at any one time the amount of combined nitrogen available to a plant is limited. Replenishment of nitrogen to a soil system is required in order to replace losses and maintain optimum levels. Significant nitrogen losses from the soil system are the result of plant uptake, leaching and denitrification. Nitrogen sources to replace these losses include man's application of fertilizer, the decay of organic materials, and biological nitrogen fixation. Without the replacement of combined nitrogen by one or more of these methods, the amount of nitrogen available to plants is limited.

Nitrogen replenishment by biological nitrogen fixation is of great importance in the temperate climatic zone. Agriculture has benefited from the Rhizobium and legume

association since early recorded time (44). Only during the past few decades, has the importance of microorganism association with nonlegumes been identified (15,62).

During this time nonlegume nitrogen fixation has been related as being important to primary plant succession, forest management, and soil conservation (3,8,18,28,37,39, 71,82).

In comparison to N_2 fixation by legumes, N_2 fixation by nonlegumes has not been investigated in depth with many plant species or with factors influencing the N_2 fixation process.

The following investigation was conducted to evaluate nitrogen fixation by additional plant species as well as the influence of temperature, and light intensity on N_2 fixation process.

CHAPTER I

LITERATURE REVIEW

Identification of Plants and Microorganisms

Plant Species Forming Root Nodules

The occurrence of nodules on nonlegume angiosperms has been recorded in botanical literature since early in the nineteenth century (49). Lists of nodule producing species have been compiled (3,8,14,44,56), with two (3,56) indicating the first known recording of nodules on each species. Since these reviews, the only additional genus to be reported producing nodules has been *Artemisia* (24). The species identified was *A. ludoviciana* Nutt.

From these surveys 119 species in 15 genera (*Alnus*, *Arctostaphylos*, *Artemisia*, *Casuarina*, *Ceanothus*, *Cercocarpus*, *Comptonia*, *Coriaria*, *Discaria*, *Dryas*, *Elaeagnus*, *Hippophae*, *Myrica*, *Purshia*, and *Shepherdia*) have been classified as nonlegumes bearing nodules.

Endophyte Inducing Nodulation

No positive identification has been made for the organism causing nodules associated with nonlegume plants. Several attempts on identification have yielded varying results (8,33,38,54,58,61,66,72,83). Isolation appears to be

the limiting step since numerous organisms have been described. Reinoculation has either (1) not been attempted; (2) been successfully attempted but never repeated; or (3) been attempted but has failed to produce a new nodule.

Actinomycetes, bacteria or fungi were isolated from nodules on nonlegumes in 30% of the trials attempted by Uemura (72). A certain type of actinomycete was regularly isolated from specific species. Several species of bacteria and fungi were isolated, but neither were isolated from a distinct plant species. Two types of actinomycetes were regularly found with the Alnus species and Elaeagnus umbellata Thunb. while one or the other of the two types was predominately associated with either Myrica, Coriaria, or Ceanothus. Reinoculation on sterile seedlings did not produce nodules when either actinomycetes, bacteria or fungi were used.

A 1941 report by von Plotho (in 72) on the successful reinoculation of an actinomycete obtained from Alnus incana (L.) Moench. onto Alnus glutinosa (L.) Gaertn. has been disputed. Sterile technique was not mentioned and reincubation has not been successfully repeated (8,54,72).

Additional investigations by Pommer (in 72), Kappel and Wartenberg (in 72), Ficuczek (in 8), Wollum et al. (83), Hoeppl and Wollum (35), Furman (26), and Silver (61) have indicated an actinomycete or streptomycete is the causal organism without successful and repeated reinoculation.

A taxonomic placement of the organism associated with nonlegumes has been proposed (7). Placement is in the Actinomycetales with 10 species in a single genus (Frankia) and a single family (Frankiaceae) which is separated from Actinomycetaceae and Streptomycetaceae.

Nodule Formation

Angulo Carmona (4) determined that the nodules formed on nonlegumes are adventitious lateral roots induced by the endophyte which subsequently enters the adventitious root. A growth regulator, possibly a cytokinin, is produced by the endophyte in order to induce root formation prior to entry. Phillips and Torrey (52) have found Rhizobium japonium to secrete high enough levels of cytokinin into a culture medium during the logarithmic phase of growth that it could stimulate mitotic division. By applying low concentrations of kinetin or 2-iso-pentenyl-adenine to roots of A. glutinosa, Rodriguez-Barrueco and Bermudez de Castro (57) were able to induce pseudonodules.

N₂ Fixation

Biochemical Process

A possible pathway for N₂ fixation within nodules on nonlegumes is illustrated in Figure I-1. Hardy et al. (30) and Bergersen (11) have reviewed nitrogenase and the reactions catalyzed by it. Nitrogenase from other organisms is a complex of proteins with one being an iron-molybdenum

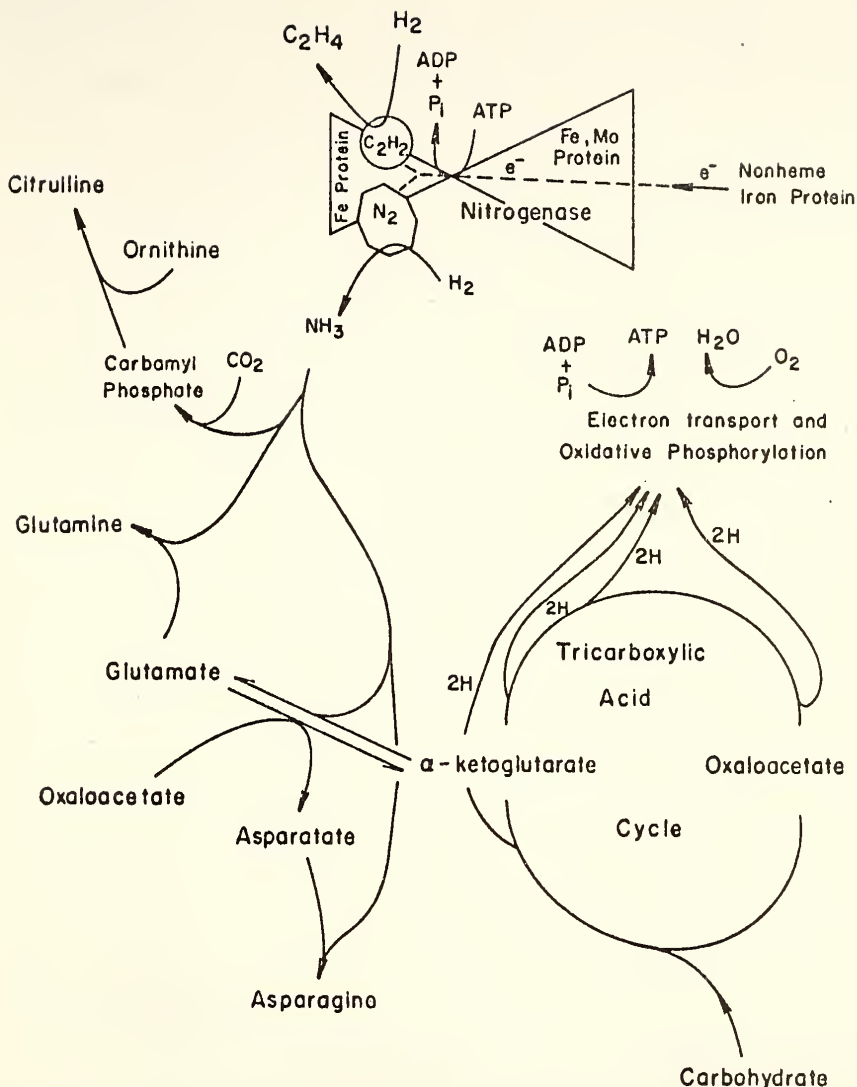


Figure I-1. A diagram of the pathways that may be involved in nitrogen fixation. The diagram was composed of information provided by Bergersen (11), Hardy et al. (30), Lehninger (41), Virtanen and Miettinen (73) and Hwang et al. (36).

protein consisting of 4 subunits and an iron protein composed of 2 subunits (53). Nitrogenase catalyzes more than one reaction at more than one site (36,53). The sites for N_2 and C_2H_2 reduction are separate (36).

Ethylene, which is the end product of C_2H_2 reduction, is not reduced by nitrogenase nor does ethylene inhibit nitrogenase activity (30). Using A. glutinosa, Virtanen and Miettinen (73) determined that N_2 was incorporated into carbamyl phosphate, citrulline, glutamic acid and ornithine soon after fixation.

Plants Investigated

Hiltner (34) and Dinger (in 4 and 8) in the 1890's using greenhouse and field studies were able to determine that nodulated A. glutinosa grew larger than plants that did not possess nodules. Not until the 1950's was it determined that the site of N_2 fixation was the nodule. By Using nodulated versus nonnodulated root systems, Bond et al. (16) and Virtanen et al. (74) determined that nodules were needed in order to have ^{15}N translocated into the tops of A. glutinosa.

By use of ^{15}N , Myrica gale L., and Hippophae rhamnoides L. (12) as well as Casuarina cunninghamiana, Ceanothus azureus Kell., and Shepherdia canadensis Nutt. (13) were shown to be N_2 fixing species. Gardner and Bond (28) established earlier that Shepherdia canadensis produced better growth and had a higher N content when nodulated than when

nonnodulated. Gardner (27) reported similar results with Elaeagnus angustifolia L. Alnus jorullensis was shown to possess N_2 -fixing nodules in a comparison of growth between nodulated and nonnodulated plants grown in a N free solution as well as with the use of ^{15}N (55). Wayle and Vlamis compared growth of nodulated plants (Purshia tridentata (Pursh.) DC and P. glandulosa Curran.) with a nonnodulated plant (lettuce) on N-deficient and N-fertilized soils. The results indicated the Purshia sp. grew well on both N-deficient and N-fertilized soil while the growth of lettuce was poor on the former and excellent on the latter. The difference between Purshia and lettuce was attributed to nitrogen fixation by the root nodules on Purshia (77). Vlamis, Schultz and Biswell found differences in the growth of Ceanothus integerrimus H. & A. (75) and Cercocarpus betuloides Nutt. (76) when grown in pots containing N-deficient soil. The best growth occurred with nodulated plants.

Twelve species of Ceanothus [incanus T. & G. integerrimus, jepsonii Greene, prostratus Benth., foliosus Parry, cuneatus (Hook.) Nutt., velutinus Dougl., thyrsiflorus Esch., gloriosus J.T. Howell, sorediatus H. & A., divaricatus Nutt., and griseus (Trel.) McMinn.] were identified as having N_2 -fixing nodules by use of ^{15}N (21). Myrica javanica and Casuarina junghuhniana were, also, identified as possessing N_2 fixing nodules (8). Growth of these plants in an N-free

medium, ^{15}N , and acetylene (C_2H_2) reduction were used to demonstrate N_2 fixation. ^{15}N and C_2H_2 reduction were, also, used to establish Myrica cerifera and Casuarina equisetifolia Forst. as possessing N_2 -fixing nodules (64). Ziegler and Huser (84) were able to show N_2 -fixing nodules occurred on Comptonia peregrina (L.) Coult. with use of ^{15}N . With one of the earliest uses of C_2H_2 reduction, Stewart et al. (70) confirmed that this species as well as Alnus rugosa (DuRoi) Spreng. possessed N_2 -fixing nodules. Fessenden et al. (25) likewise confirmed N_2 fixation by C. peregrina when they compared ^{15}N with C_2H_2 reduction as a means of determining N_2 fixation. By use of C_2H_2 reduction, nodules from Alnus glutinosa, A. crispa (Ait.) Pursh., Elaeagnus angustifolia, E. umbellata, Hippophae rhamnoides, Myrica pensylvanica Lois., Casuaria cunninghamiana, Shepherdia argentea Nutt., S. canadensis and Cercocarpus montanus Raf., have also been found to be N_2 -fixing plants (20,35,46,50,63).

Acetylene Reduction

Development

Acetylene (C_2H_2) was determined to be a competitive inhibitor of N_2 fixation by Schollhorn and Burris (59) as well as by Dilworth (22). Dilworth (22) in turn established ethylene (C_2H_4) as the reduction product of C_2H_2 . Recently Hwang et al. (36) determined that more than one reduction site was located on the nitrogenase complex. Later, it was found that C_2H_2 was a noncompetitive

inhibitor of N_2 fixation. From the previous work Hardy and Knight (32) established the C_2H_2 reduction assay which utilized the conversion of C_2H_2 to C_2H_4 by the nitrogenase possessed by the N_2 -fixing species and the monitoring of the changes in gas volume by gas chromatographic technique. A recent review (30) summarizes information on the C_2H_2 reduction assay, and presents this information on a variety of N_2 fixing systems in table form.

The C_2H_2/N_2 Conversion Factor

Acetylene versus N_2 reduced, theoretically will be 3 to 1. This occurs because N_2 to NH_3 requires 6 electrons, whereas C_2H_2 to C_2H_4 requires only 2 (60).

Values obtained from nodules of different species varied as indicated in Table I-1.

Replacement of N_2 in the C_2H_2 Assay

No difference occurs in rate of C_2H_2 reduction by non-legume nodules whether N_2 is included with the sample or is replaced with an inert gas (25,43). Sloger (64), however, reported a reduction in C_2H_2 -reducing activity when N_2 was present.

Table I-1. The ratio acetylene reduced to nitrogen fixed.

Investigator	Plant Material	C_2H_2/N_2
Mague and Burris (43)	<u>Glycine max</u>	0.9 to 3.1
Bergersen (10)	<u>Glycine max</u>	2.7 to 3.9
Fessenden et al. (25)	<u>Comptonia peregrina</u>	2.84 to 3.44
Akkermans (1)	<u>Alnus glutinosa</u>	1.1 to 3.4
Stewart et al. (70)	<u>Alnus rugosa</u>	3.26 (calculated from Figure 7)
Russell and Evans (in 30)	<u>Alnus rugosa</u>	2.0 to 2.8

CHAPTER II

EFFECT OF TEMPERATURE ON C_2H_2 REDUCING ACTIVITY ASSOCIATED WITH ROOT NODULES ON WOODY TREES AND SHRUBS

Introduction

Observations by McNiel and Carpenter (45) have shown that N_2 fixation is influenced by temperature and also, that the influence of temperature varies with different plant species. The fact that temperature does affect N_2 fixation or C_2H_2 reduction associated with nodulated plants has been previously reported by many other investigators (1,5,9,25, 78,79).

Several investigators have determined that the optimum temperature for N_2 fixation of some agronomic legumes to be between 20 C and 25 C (5,30,69,73). Temperatures above or below this range have caused a reduction in the amount of C_2H_2 or N_2 reduced by the nodule (5,30,69,73). Variations in reduction activity have occurred with various strains of microorganisms or between plant species when plants or nodules are treated at any one temperature. The variation among the various species holds through more than one temperature level (29,51).

Apparently the first attempt to characterize the influence of temperature on nitrogen fixation by a nonlegume

was with Alnus viridis DC. (9). The optimum temperature for nitrogen fixation was 20 C with reduced activity occurring at temperatures of 15 C or 25 C.

Wheeler (80) a year later reported an optimum reduction rate with Alnus glutinosa L. Gaertn. to occur at 25 C. Transforming his data into an Arrhenius plot indicates that it is biphasic, but insufficient treatments did not permit determination of a specific temperature for a change in activation energy. Waughman (78), though not determining a rate between 20 C and 30 C, established that the maximum reduction rates for A. glutinosa and Hippophae rhamnoides L. occurred at 20 C. The rate was reduced from this high level at 15 C and 30 C. The Arrhenius plot for this data indicated no change in activation energy between 5 C and 20 C. Akkermans (1), also working with A. glutinosa and H. rhamnoides established that the maximum reduction rate to have occurred at 20 C. By transferrring the data obtained from the above plants to an Arrhenius plot, it proved to be biphasic. The change in activation energy occurred at 15 C for A. glutinosa and 11 C for H. rhamnoides. Fessenden et al. (25) working with Myrica asplenifolia (Comptonia peregrina) (L.) Coult. established 30 C as the temperature for maximum nitrogen fixation. This is the highest temperature reported for either a legume or a nonlegume to be fixing nitrogen at an optimum rate. Their Arrhenius plot was biphasic with the change in activation energy occurring at 11 C.

Due to a lack of information on other species and to determine if treatment of the plant with intact nodules would differ from treatment of excised nodules, the following temperature studies were carried out.

Materials and Methods

Experiments

Four different experiments were established in order to determine the influence of temperature on N_2 fixation.

Experiment 1.

To determine the N_2 -fixation rate of nodules from Elaeagnus umbellata Thunb. as affected by four different temperatures when treating only the plant root zone and not the tops.

Experiment 2

To compare the N_2 -fixation rate of nodules from E. umbellata when either exposing the plant roots with nodules (Part A) or when exposing only excised nodules (Part B) to the treatment temperature.

Experiment 3

To determine the effect of four different temperatures on the N_2 -fixation rate using excised nodules from A. glutinosa, a nonlegume, and Robinia pseudoacacia L., a legume.

Experiment 4.

To determine the temperature coefficient (Q_{10}) for nine species using excised nodules.

Plant Material

Whole plants used in experiments 1 and 2 were bareroot seedlings of E. umbellata obtained from a midwest nursery. The 30 to 45 cm plants were potted into a 15 x 18 cm container using a peat and perlite (50:50 by volume) medium and grown for at least 6 weeks before treatment. The excised nodules from E. umbellata, A. glutinosa or R. pseudoacacia used in experiments 1, 2, 3 or 4 were obtained from plants started from seed in a greenhouse, then field grown, and were 2 to 4 years in age when nodules were removed for analysis. The soil at the growing site was a sandy loam possessing a pH of 6.5.

The excised nodules from Alnus rugosa (DuRoi) Spreng., Comptonia peregrina (L.) Coult., Elaeagnus commutata Bernh., E. angustifolia L., Hippophae rhamnoides L. and Shepherdia argentea Nutt. used in experiment 4 were grown as stated for the 3 previous species except these 6 species were 1 year old.

Nodule Preparation

In experiments 1, 2 and 3, nodules were excised for acetylene reduction analysis and the nodules from individual plants were analyzed separately. Each nodule was divided

into segments with the lobes being removed and assayed while the woody basal portion was discarded. The lobes were washed under tap water, blotted dry on paper towels, and their fresh weight determined prior to their placement into an analysis flask.

Nodules used in experiment 4 were removed from one or more plants of each of the 9 species. Nodules were prepared in the same manner as described in the previous paragraph except that enough lobes were combined from several plants in order to provide sufficient material for all treatments and replications.

Temperature Treatments

In experiment 1 the root zones of one year old, potted E. umbellata were placed in a chamber that was adjusted to the following temperatures: 26-28 C, 13-14 C, 8-9 C, and 2-4 C. The use of this chamber permitted the root zone to be maintained at these temperatures while the tops of the plants were held at 26-28 C. Plants were treated on five different dates with five plants per date for each temperature. All treatments were made at one temperature before changing the chamber and treating at another temperature level.

Fluorescent lighting was used to provide 1600 foot-candles of light for 16 hours in each day. Nodules were removed during the 4th to 6th hour of light. The plants were subjected to the temperature treatment for approximately

36 hours before the nodules were detached. Nodule samples were obtained from each of five plants used per treatment each day and determinations were made on 5 different days for each temperature range.

Between 10 and 15 minutes elapsed while nodules were separated, cleaned, placed in a flask, and exposed to acetylene. Then each completed flask was returned for one hour to the temperature in which the root zone of the plant had been previously exposed.

In experiment 2, part A, half of the plants were treated as explained for experiment 1, except only a temperature of 2-4 C was used. In part B, the other half of the plants used were field grown. The nodules were detached from the plant in the field and transported to a lab for processing and analysis. Approximately 60 minutes elapsed between digging the plant, separating the nodule, cleaning, placing the lobes in a flask and exposing to acetylene. The excised nodules were exposed to 2-4 C for one hour. Five samples, each from a different plant were prepared on 5 different dates.

In experiment 3, excised nodules from field grown Alnus glutinosa and Robinia pseudoacacia were prepared as were the nodules in part B of experiment 2 except for the temperature treatment. As in experiment 1, four temperature treatments (26-28, 13-14, 8-9, and 2-4 C) were used. Five samples, each from a different plant, were prepared on 5 different dates.

In experiment 4, water or ice baths were used to establish treatment temperatures at 0 C, 10 C, 20 C, 30 C, and 40 C. At each temperature five tubes containing nodules prepared as described previously under nodule preparation were incubated for one hour. The following species were used: Alnus glutinosa, A. rugosa (DuRoi) Spreng., Comptonia peregrina, Elaeagnus angustifolia L., E. commutata Bernh., E. umbellata, Hippophae rhamnoides, Shepherdia argentea Nutt., Robinia pseudoacacia. The Q_{10} was determined by dividing the mean value of ethylene produced at one temperature into the quantity produced at 10 C higher.

Measurement of Acetylene Reduction

Assays on nodules in experiments 1, 2 and 3 were made in glass 265 ml volumetric flasks capped with a rubber stopple. Each flask contained approximately 0.5 g of nodule. A 12 ml Monoject disposable syringe was used to remove 12 ml of air and replaced with 12 ml of commercial grade acetylene. At the end of a one hour incubation period for each treatment, a 1 ml sample was removed with a 1 ml disposable syringe and analyzed. Analysis of the acetylene and ethylene was completed with a Varian 1200 gas chromatograph equipped with a flame-ionization detector (Appendix). The column contained Porapak R and was held at 40 C. Sample peak heights of ethylene were compared to a standard curve prepared daily from a known ethylene concentration.

Assays for the determinations in experiment 4 were made in 17 ml culture tubes containing 0.1 g nodule and capped with a rubber stopple. The removal of air and the introduction of acetylene was in 1 ml quantities using a 1 ml disposable syringe. The remainder of the analysis was as described above.

The data obtained were transformed to $\log(X + 1)$ prior to carrying out an analysis of variance (65).

Results and Discussion

Experiment 1

Temperature influence on C_2H_2 reduction by nodules from E. umbellata when exposing the root zone and not the plant top to various temperature is presented in Table II-1. Very little activity occurred at 2-4 C. The ethylene production at this temperature may be due to the higher nodule temperature at the start of the analysis before temperature uniformity occurs.

As the temperature increased to 13-14 C, the amount of C_2H_4 increased with significant increases between 2-4 C and 8-9 C as well as between 8-9 C and 13-14 C. Other nonlegumes have been reported to have similar increases in reducing activity with increases in temperature (1,9,25,78).

No significant increase in C_2H_4 production occurred with the increase in temperature from 13-14 C to 26-28 C. Akkermans (1) obtained similar results at 12 C and 26 C.

Table II-1. The effect of temperature on acetylene reduction by nodules from *E. umbellata* when exposing the root zone with nodules to each temperature.

Temperature (°C)	χ_T^z $\log[(\mu\text{l C}_2\text{H}_4/\text{g nodule/hr})+1]$	χ^y $\mu\text{l C}_2\text{H}_4/\text{g nodule/hr}$
2-4	.34207c ^x	3.5
8-9	1.39068b	46.3
13-14	2.17908a	182.6
26-28	2.24364a	182.4

^zMean of $\log[(\mu\text{l C}_2\text{H}_4/\text{g nodule/hr})+1]$ for 5 replications with 5 subsamples.

^yMeans of $\mu\text{l C}_2\text{H}_4/\text{g nodule/hr}$ for 5 replications with 5 subsamples.

^xMean separation within column by LSD, 1% level.

However, Benecke (9), Fessenden et al. (25), and Waughman (78), indicate an additional increase between these temperatures for other nonlegumes. The results obtained in this study may be due to moisture stress during the 36 hour treatment period at the higher temperature.

Experiment 2

The comparison in C_2H_2 reduction by nodules from E. umbellata when exposing either the plant root zone with nodules or only excised nodules to 2-4 C is presented in Table II-2. No significant difference occurred at this temperature.

The limiting factor to reducing activity at this temperature, whether a photosynthesis product, an enzyme, or other factor was not determined. When either the root zone with nodules or only excised nodules are treated at 2-4 C, no difference in the C_2H_2 -reducing rate occurred.

Experiment 3

The effect of 4 different temperatures on the C_2H_2 -reduction rate using excised nodules from A. glutinosa and R. pseudoacacia is presented in Tables II-3 and II-4.

Each species responded differently to the temperature treatment. In both cases there was a large increase in the amount of C_2H_4 produced between the low and high temperature.

Table II-2. Comparison in C_2H_2 -reducing rate at 2-4 C between exposing the root zone plus nodules and exposing only excised nodules of E. umbellata to the temperature.

Material treated	$\mu l C_2H_4/g \text{ nodule/hr}$
nodules when root zone was treated	3.5 ^{zy}
only excised nodules treated	5.3

^zMeans for 5 replications with 5 subsamples.

^yMeans were not significantly different at 5% level.

Table II-3. The effect of temperature on acetylene reduction when using excised nodules from Alnus glutinosa.

Temperature (°C)	$\bar{x}_T^z$ $\log[(\mu\text{l C}_2\text{H}_4/\text{g nodule/hr})+1]$	$\bar{x}^y$ $\mu\text{l C}_2\text{H}_4/\text{g nodule/hr}$
2-4	.31638c ^x	1.98
8-9	.19544c	1.16
13-14	.77314b	8.42
26-28	2.02900a	120.26

^zMean of $\log[(\mu\text{l C}_2\text{H}_4/\text{g nodule/hr})+1]$ for 5 replications with 5 subsamples

^yMeans of $\mu\text{l C}_2\text{H}_4/\text{g nodule/hr}$ for 5 replications with 5 subsamples.

^xMean separation within column by LSD, 5% level.

Table II-4. The effect of temperature on acetylene reduction when using excised nodules from R. pseudoacacia.

Temperature (°C)	X_T^Z	X^Y
	$\log[(\mu\text{l C}_2\text{H}_4/\text{g nodule/hr})+1]$	$\mu\text{l C}_2\text{H}_4/\text{g nodule/hr}$
2-4	.55770c ^x	3.7
8-9	1.04857b	14.6
13-14	1.26867b	22.3
26-28	1.93412a	102.4

^ZMean of $\log[(\mu\text{l C}_2\text{H}_4/\text{g nodule/hr})+1]$ for 5 replications with 5 subsamples.

^YMeans of the $\mu\text{l C}_2\text{H}_4/\text{g nodule/hr}$ for 5 replications with 5 subsamples.

^xMean separation within column by LSD, 5% level.

Nodules from A. glutinosa did not show a significant increase in C_2H_4 production between 2-4 C and 8-9 C while the increase was significant between 8-9 C and 13-14 C as well as between 13-14 C and 26-28 C. This indicates A. glutinosa may not reduce acetylene or fix nitrogen over lower temperatures (less than 13-14 C). This is similar to results reported by Akkermans (1) who also reported a level of C_2H_4 production approximately 1/4 the value of that for 26-28 C in Table II-3. Part of the difference may be due to age of plant. The Alnus investigated by Akkermans (1) was 10 years old versus 4 years for the plants in this investigation.

Nodules from R. pseudoacacia did not show a significant increase in C_2H_4 production between 8-9 C and 13-14 C. However, the increase was significant between 2-4 C and 8-9 C as well as between 13-14 C and 26-28 C. No published data is available on factors influencing N_2 fixation for any Robinia species. The reducing activity for other legumes, however, does increase with increasing temperature (29,30).

Experiment 4

The temperature coefficients determined for 9 different plant species are presented in Tables II-5 through II-13. For each plant species, the Q_{10} values between 10 C and 20 C indicate that a reaction is taking place that requires a high activation energy for an enzymatic process.

Table II-5. Temperature coefficients (Q_{10}) for C_2H_2 reduction to C_2H_4 for nodules from Alnus glutinosa.

Temperature (°C)	$\mu l C_2H_4/g \text{ nodule/hr}^z$	Q_{10}
0	0.1	
10	3.5	
		8.2
20	28.8	
		4.26
30	122.6	
		0.22
40	26.8	

^zValues are means of 5 replications.

Table II-6. Temperature coefficients (Q_{10}) for C_2H_2 reduction to C_2H_4 for nodules from Alnus rugosa.

Temperature (°C)	$\mu l\ C_2H_4/g\ nodule/hr^Z$	Q_{10}
10	10.0	9.8
20	97.7	2.1
30	204.7	0.46
40	95.2	

^ZValues are means of 5 replications.

Table II-7. Temperature coefficients (Q_{10}) for C_2H_2 reduction to C_2H_4 for nodules from Comptonia peregrina.

Temperature (°C)	$\mu l C_2H_4/g \text{ nodule/hr}^Z$	Q_{10}
0	0.2	
10	2.8	
		6.2
20	17.4	
		3.2
30	55.7	
		0.6
40	34.6	

^ZValues are means of 5 replications.

Table II-8. Temperature coefficients (Q_{10}) for C_2H_2 reduction to C_2H_4 for nodules from Elaeagnus angustifolia.

Temperature (°C)	$\mu l C_2H_4/g \text{ nodule/hr}^Z$	Q_{10}
10	3.4	
		8.77
20	30.2	
		2.56
30	77.3	
		1.18
40	91.3	

^ZValues are means of 5 replications.

Table II-9. Temperature coefficients (Q_{10}) for C_2H_2 reduction to C_2H_4 for nodules from Elaeagnus commutata.

Temperature (°C)	$\mu l\ C_2H_4/g\ nodule/hr^Z$	Q_{10}
0	1.00	
10	20.7	5.6
20	116.6	2.95
30	344.5	0.58
40	201.1	

^ZValues are means of 5 replications.

Table II-10. Temperature coefficients (Q_{10}) for C_2H_2 reduction to C_2H_4 for nodules from Elaeagnus umbellata.

Temperature (°C)	$\mu l\ C_2H_4/g\ nodule/hr^z$	Q_{10}
0	0.14	
10	23.00	
		6.93
20	159.4	
		2.18
30	348.8	
		0.84
40	291.6	

^zValues are means of 5 replications.

Table II-11. Temperature coefficients (Q_{10}) for C_2H_2 reduction to C_2H_4 nodules from Hippophae rhamnoides.

Temperature (°C)	$\mu l C_2H_4/g \text{ nodule/hr}^Z$	Q_{10}
0	0.4	
10	6.6	
		17.8
20	117.7	
		1.0
30	117.2	
		0.15
40	17.0	

^ZValues are means of 5 replications.

Table II-12. Temperature coefficients (Q_{10}) for C_2H_2 reduction to C_2H_4 for nodules from Robinia pseudoacacia.

Temperature (°C)	$\mu l C_2H_4/g \text{ nodule/hr}^Z$	Q_{10}
0	0.1	
10	8.1	
		15.5
20	126.1	
		1.1
30	143.4	
		0.2
40	30.8	

^ZValues are means of 5 replications.

Table II-13. Temperature coefficients (Q_{10}) for C_2H_2 reduction to C_2H_4 for nodules from Shepherdia argentea.

Temperature (°C)	$\mu l C_2H_4/g \text{ nodule/hr}^Z$	Q_{10}
0	0.14	
10	9.9	
		4.0
20	39.6	
		3.6
30	143.0	
		0.63
40	90.0	

^ZValues are means of 5 replications.

For temperatures above 20 C differences occurred between species. The amount of C_2H_4 produced by nodules from A. glutinosa, A. rugosa, C. peregrina, E. commutata, E. umbellata, and S. argentea (Figures II-1 to II-6) reached a maximum level at 30 C. Fessenden et al. (25) had reported C_2H_2 reduction to be maximum at 30 C for C. peregrina but Akkermans (1) and Waughman (78) had reported maximum reduction rates to occur at 20 C for other nonlegumes. For the seven species with maximum reduction rates at 30 C, the Q_{10} between 20 C and 30 C indicates an enzymatic reaction is still occurring but at a reduced rate compared to the lower temperatures (Tables II-5 through II-7, II-9, II-10 and II-13). The inserts in Figure II-1 through Figure II-6 give Arrhenius plots of the data for each species. The Arrhenius plots are biphasic. This indicated a change in activation energy had occurred. The approximate temperature associated with the change in activation energy for each species is presented in Table II-14. Fessenden et al. (25) determined the change in activation energy to have occurred at 11 C for C. peregrina.

The amount of C_2H_4 produced by nodules from H. rhamnoides (Figure II-7) and R. pseudoacacia (Figure II-8) reached a maximum level between 20 C and 30 C. Waughman (78) and Akkermans (1) both reported 20 C as the optimum temperature for C_2H_2 reduction by nodules from H. rhamnoides. Hardy et al. (31) determined the optimum temperature for C_2H_2 reduction by a legume to be between 20 C and 30 C.

Figure II-1. The effect of various incubation temperatures on C_2H_4 production by nodules from Alnus glutinosa. The vertical bars represent $\pm$ the standard error of the mean. The Arrhenius plot of the same data is included in the insert.

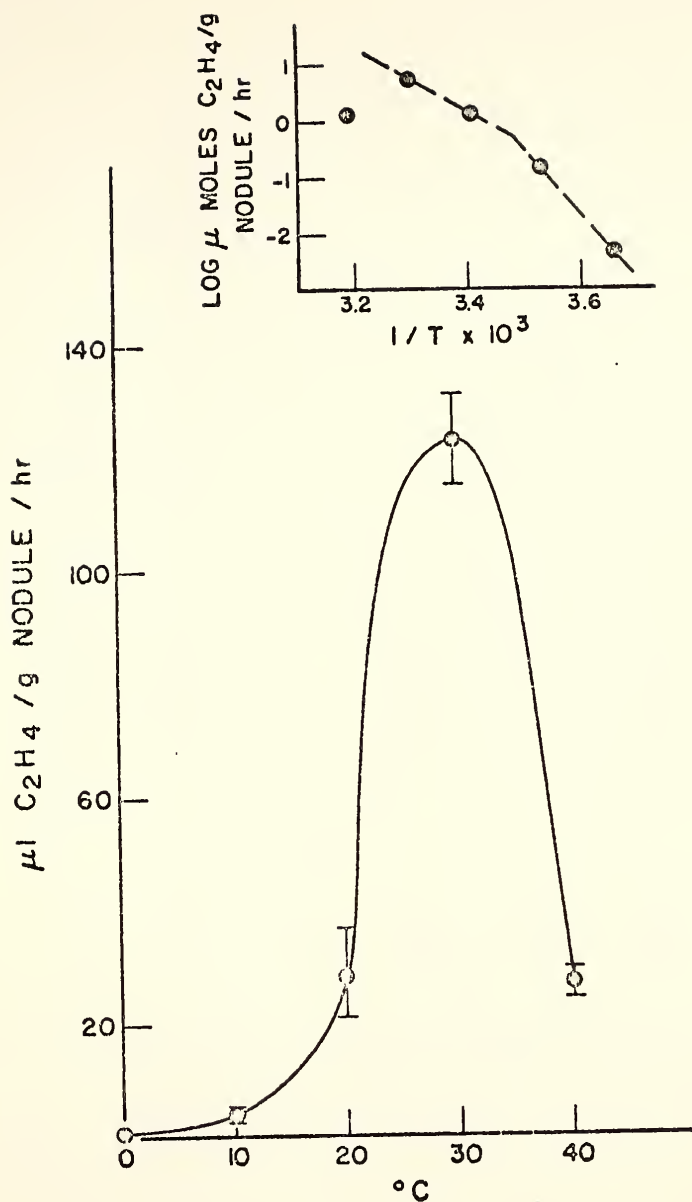


Figure II-1.

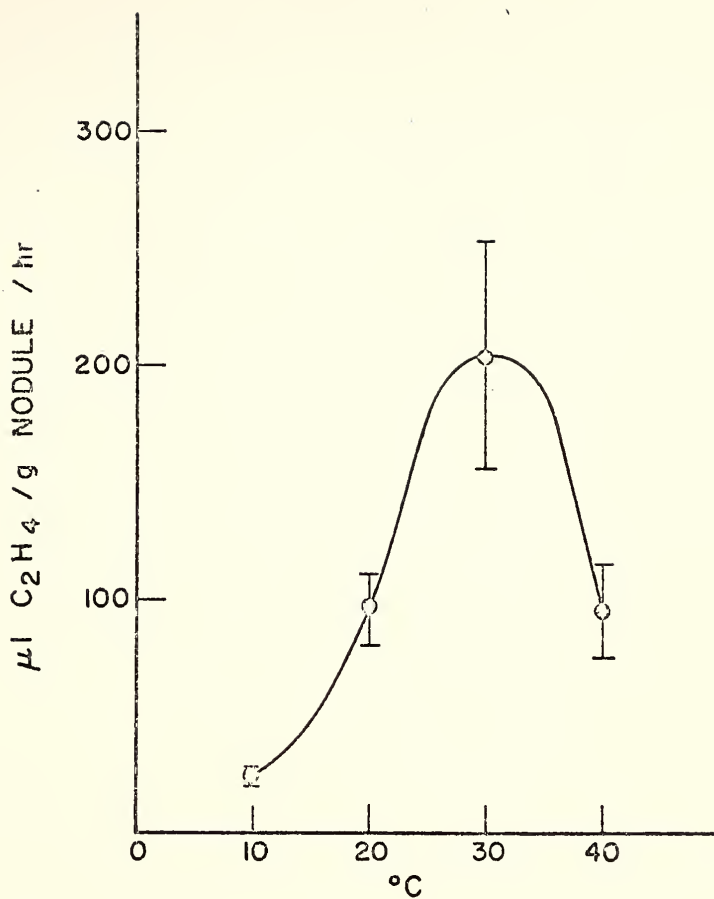


Figure II-2. The effect of various incubation temperatures on C_2H_4 production by nodules from *Alnus rugosa*. The vertical bars represent $\pm$ the standard error of the mean.

Figure II-3. The effect of various incubation temperatures on C_2H_4 production by nodules from Comptonia peregrina. The vertical bars represent $\pm$ the standard error of the mean. The Arrhenius plot of the same data is included in the insert.

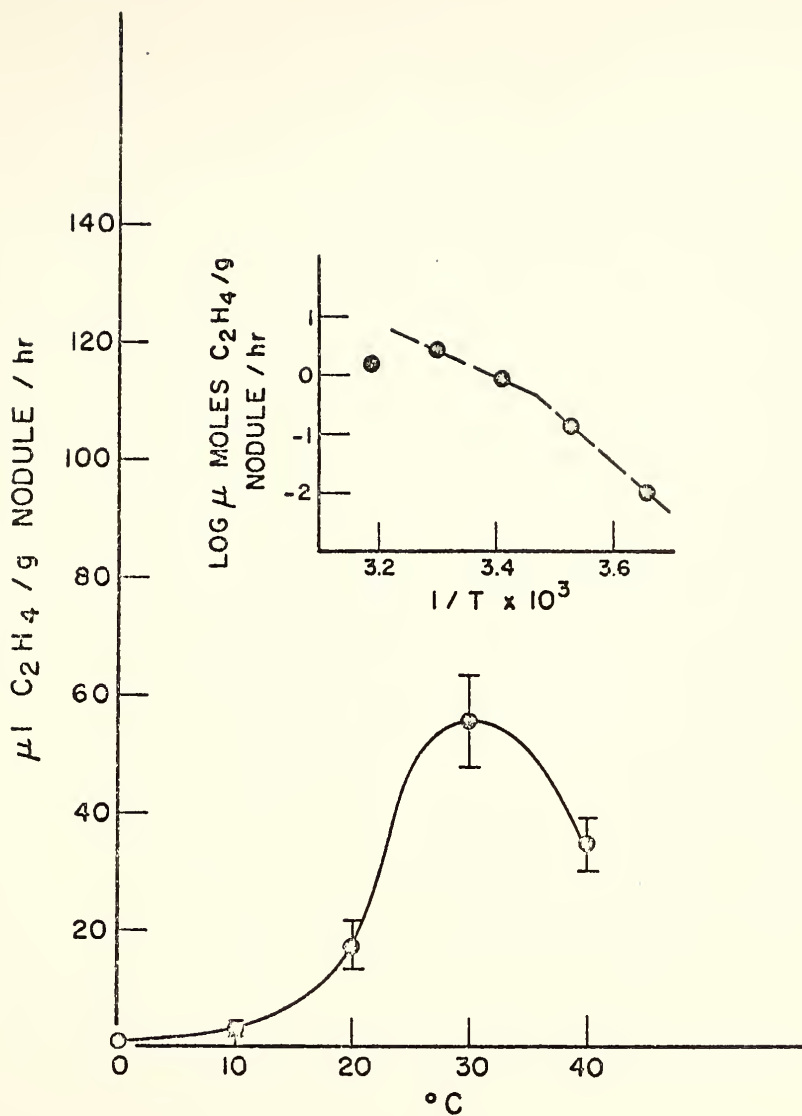


Figure II-3.

Figure II-4. The effect of various incubation temperatures on C_2H_4 production by nodules from Elaeagnus commutata. The vertical bars represent $\pm$ the standard error of the mean. The Arrhenius plot of the same data is included in the insert.

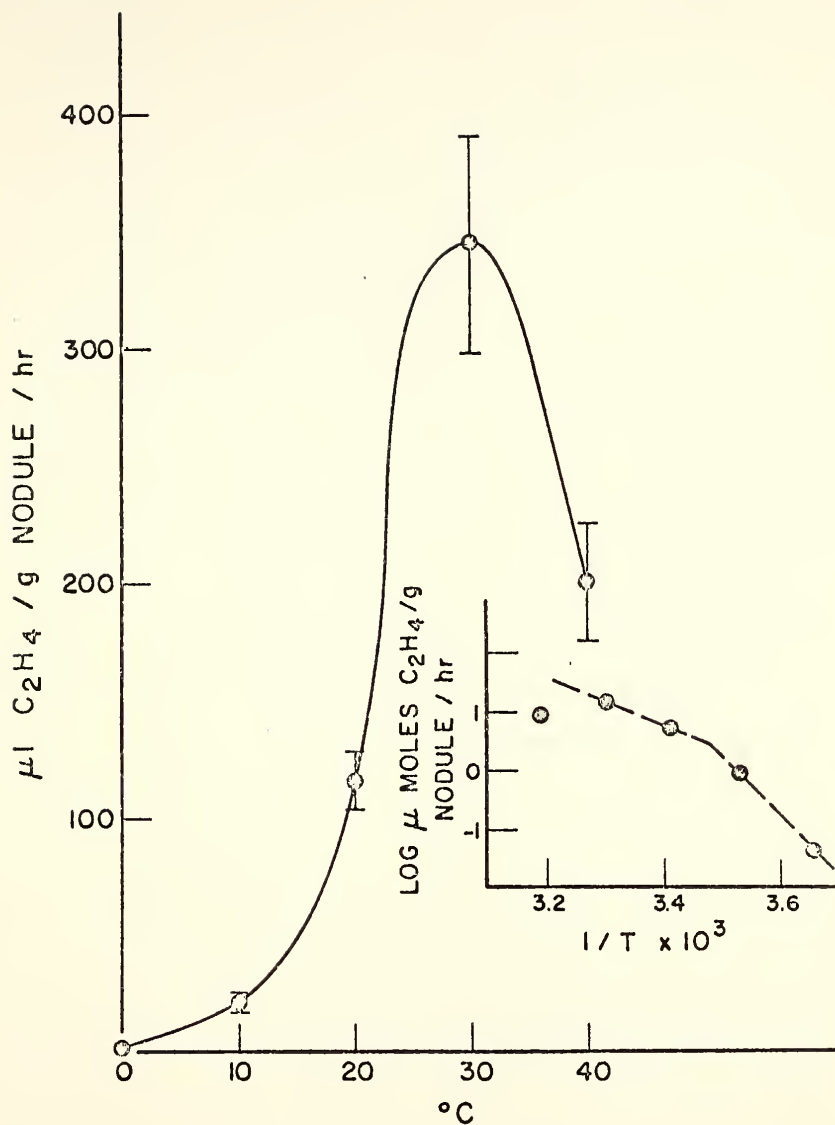


Figure II-4.

Figure II-5. The effect of various incubation temperatures on C_2H_4 production by nodules from Elaeagnus umbellata. The vertical bars represent $\pm$ the standard error of the mean. The Arrhenius plot of the same data is included in the insert.

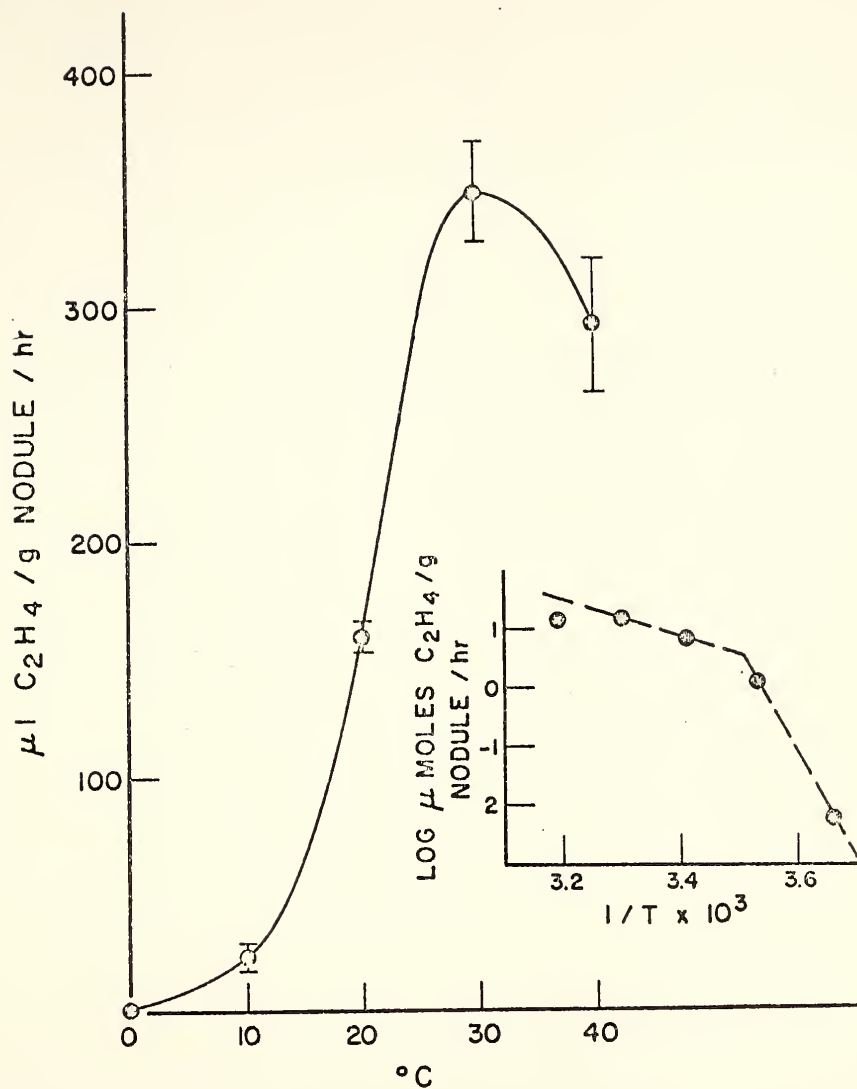


Figure II-5.

Figure II-6. The effect of various incubation temperatures on C_2H_4 production by nodules from Shepherdia argentea. The vertical bars represent $\pm$ the standard error of the mean. The Arrhenius plot of the same data is included in the insert.

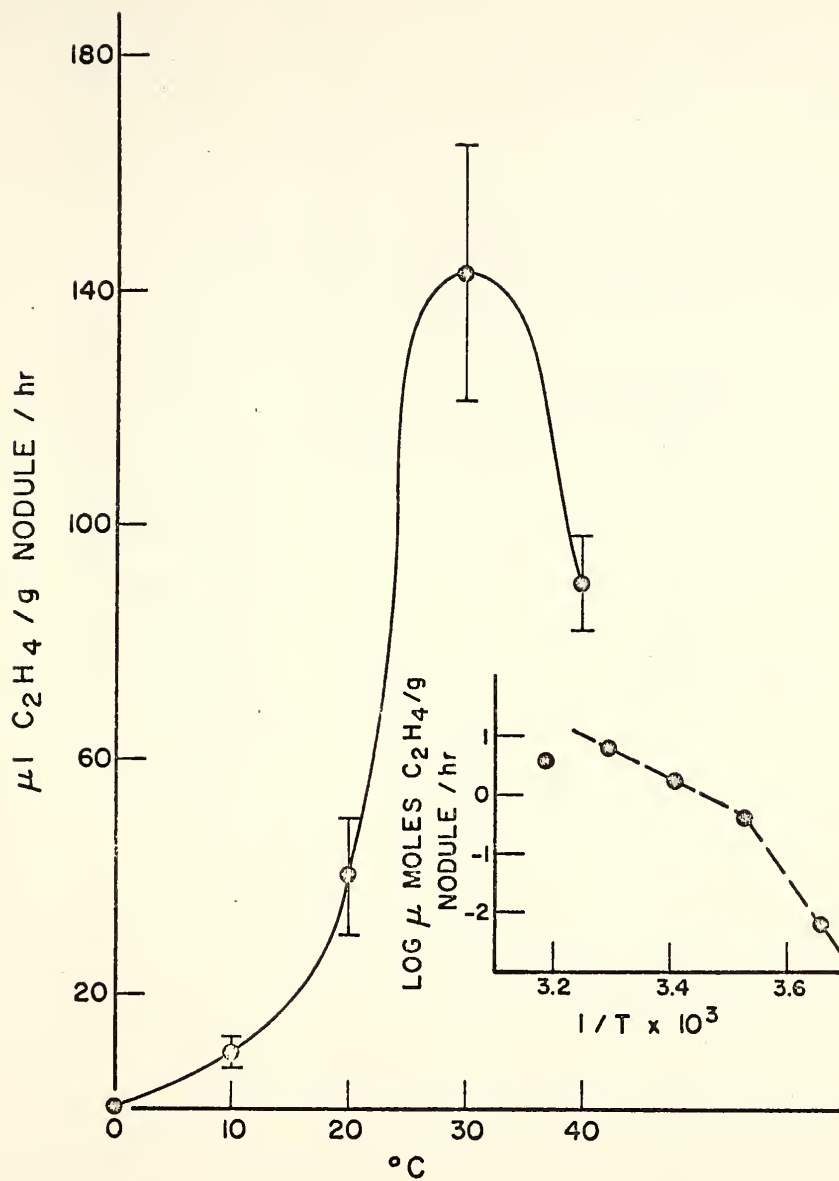


Figure II-6.

Table II-14. Approximate temperature where a change in activation energy occurs with C_2H_2 reduction to C_2H_4 by nodules from seven plant species.

Plant	Temperature (°C)
<u>A. glutinosa</u>	14
<u>C. peregrina</u>	15
<u>E. commutata</u>	14
<u>E. umbellata</u>	12
<u>H. rhamnoides</u>	20
<u>R. pseudoacacia</u>	20
<u>S. argentea</u>	11

Figure II-7. The effect of various incubation temperatures on C_2H_4 production by nodules from Hippophae rhamnoides. The vertical bars represent $\pm$ the standard error of the mean. The Arrhenius plot of the same data is included in the insert.

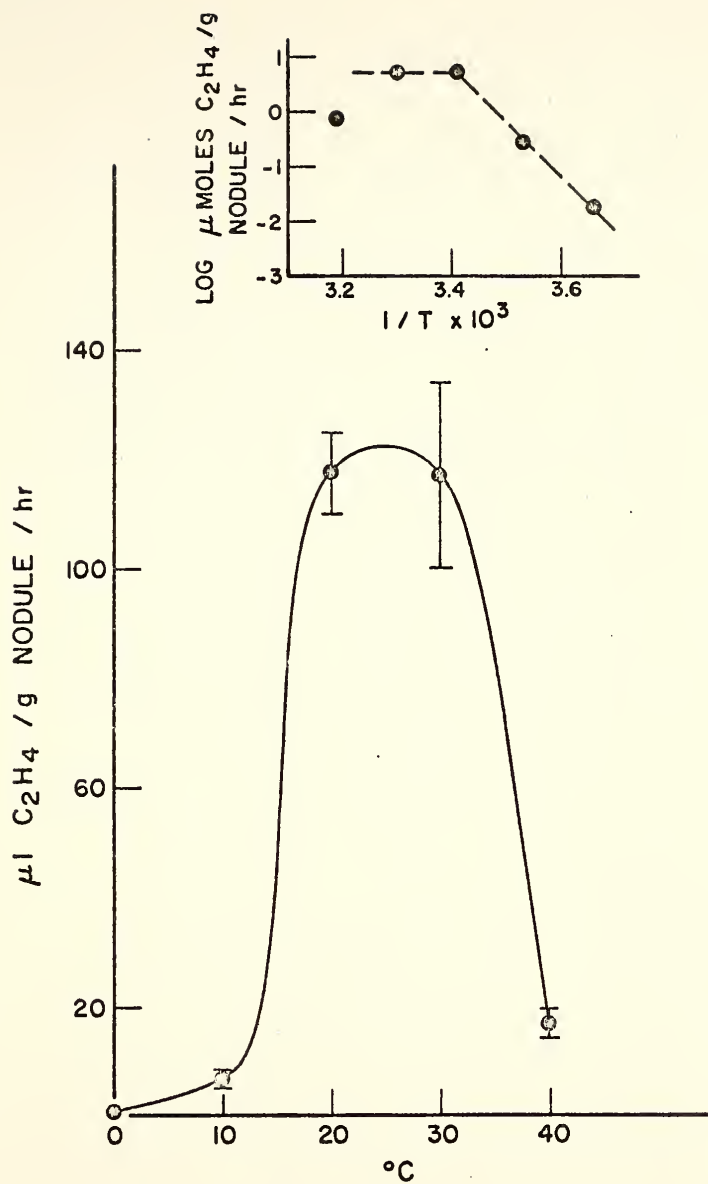


Figure II-7.

Figure II-8. The effect of various incubation temperatures on C_2H_4 production by nodules from Robinia pseudoacacia. The vertical bars represent $\pm$ the standard error of the mean. The Arrhenius plot of the same data is included in the insert.

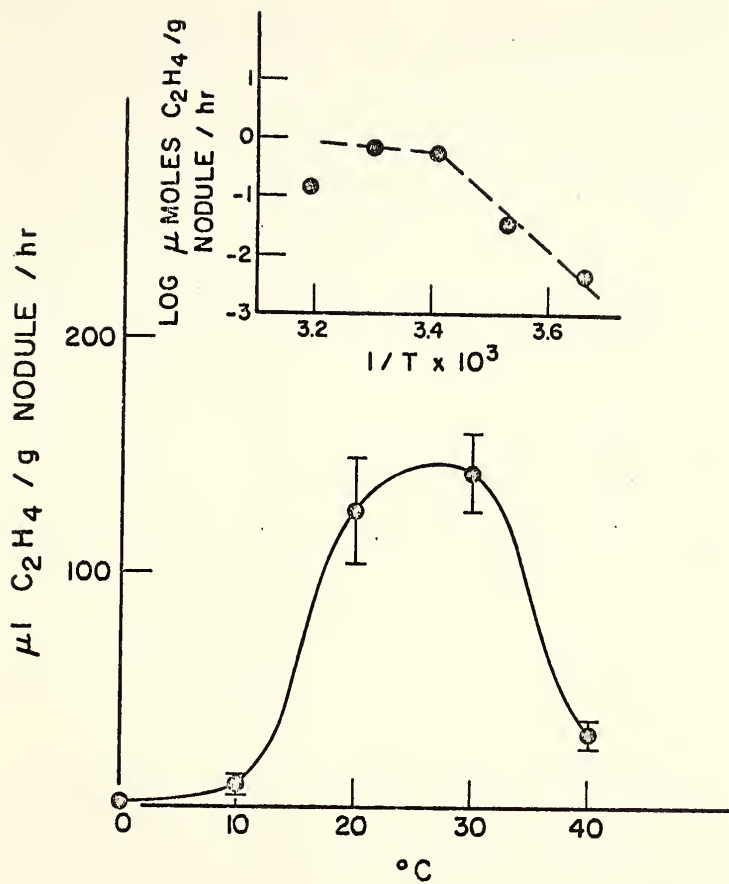


Figure II-8.

For both species, the Q_{10} between 20 C and 30 C does not indicate an accelerated enzymatic reaction (Tables II-11 and II-12).

The Arrhenius plots for H. rhamnoides (Figure II-7) and R. pseudoacacia (Figure II-8) are biphasic. The change in activation energy occurred at 20 C for both species. Waughman determined the change in activation energy to occur at 20 C. H. rhamnoides appears to be an exception for nonlegumes since the temperature associated with a change in the activation energy is 5 C to 9 C higher than for the other species studied.

The amount of C_2H_4 produced by nodules from E. angustifolia reached a maximum at 40 C (Figure II-9). This is exceedingly high compared to the other species. When comparing standard error of the mean for the means at 30 C and 40 C, there may not be any significant difference between these two readings.

For the other eight species, 40 C gave a reduction in C_2H_4 produced (Tables II-5 through II-7 and II-9 through II-13). At 40 C enzymes may be denatured (41). With a loss in enzyme activity there would be a corresponding reduction in C_2H_2 -reducing activity.

Ethylene production from nodules using E. umbellata was permanently lowered when the nodules were incubated at 40 C. Nodules incubated for 1 hour at 40 C and then exposed to C_2H_2 and incubated for an additional hour at 30 C,

Figure II-9. The effect of various incubation temperatures on C_2H_4 production by nodules from Elaeagnus angustifolia. The vertical bars represent $\pm$ the standard error of the mean.

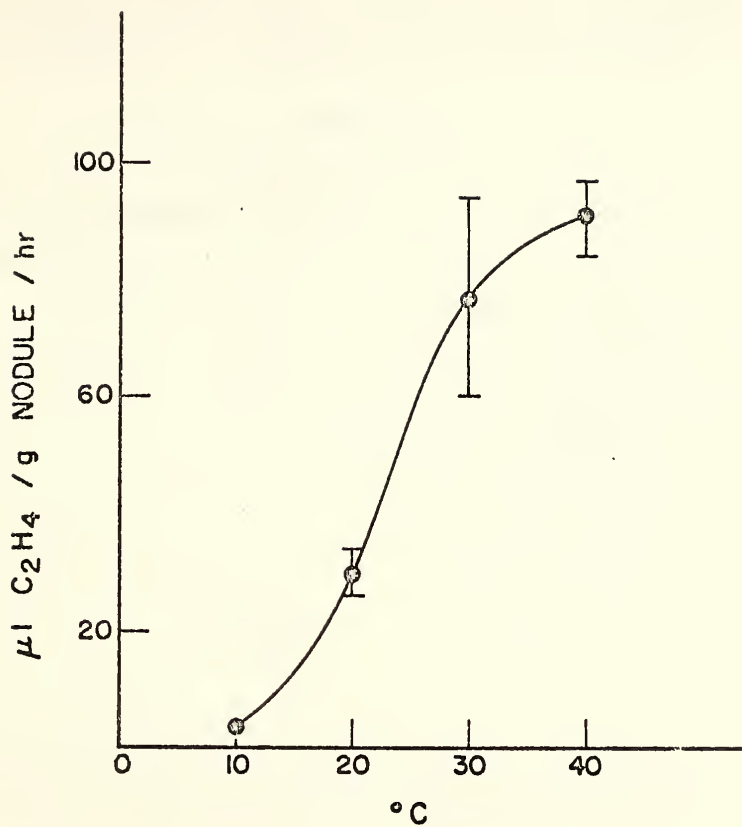


Figure II-9.

produced C_2H_4 equal to that produced by nodules treated for only 1 hour at 40 C. In both cases the quantity of C_2H_4 produced was less than the amount produced by nodules treated for 1 hour at 30 C (Table II-15). This reaffirms that damage to cell components may have occurred to 40 C.

Conclusions

Nine plant species have been characterized as to the effect of temperature on the C_2H_2 -reducing activity by nodules removed from their root systems.

Of the 9 species, 6 (A. rugosa, E. angustifolia, E. commutata, E. umbellata, S. argentea, and R. pseudoacacia) have not been characterized as to the effect of temperature on C_2H_2 -reducing activity by other investigators. Of those species studied, nonlegume nodules with the exception of H. rhamnoides have a maximum C_2H_2 -reduction rate near 30 C. H. rhamnoides and the legume, R. pseudoacacia, have a maximum C_2H_2 -reduction rate around 20 C. At temperatures near freezing, the reducing rate is very low. With temperatures above 30 C the reducing rate is again reduced and this is attributed to the destruction of some enzyme systems.

Table II-15. The effect of temperature on C_2H_2 reduction to C_2H_4 in order to determine if it is reversible.

Temperature (°C)	Incubation Time (hr)	μl C_2H_4 /g nodule/hr	Q_{10}
30	1	362.8 ^z	
40	1	115.5	.32
40+30	1+1	98.9	.27

^zValues are means of 5 replications.

CHAPTER III

FACTORS INFLUENCING THE REDUCTION OF ACETYLENE
BY EXCISED ROOT NODULES FROM
ELAEAGNUS UMBELLATA 'CARDINAL'

Introduction

Elaeagnus umbellata Thunb. was introduced into the United States about 1830, but it was not until 1963 that the 'Cardinal' selection was named and introduced (2). It is native to an area from Afghanistan and the Himalayas to Japan and Korea (2). In the northeastern and north central U.S., E. umbellata has been primarily used in plantings for wildlife, but due to its form, foliage and fruit it has potential as a valuable landscape plant. Because of its size, 4 m, it may adapt better to open landscape settings rather than small residential landscapes.

Nodules on E. umbellata were identified first in 1933 (in 56). No definite identification has been made on the causal organism. Becking (7) has proposed Frankia elaeagni of the Actinomycetales as the causal organism without positive identification. Actinomycetes have been isolated from root nodules of E. umbellata (72) and described, but re-infection was not successful.

C_2H_2 reduction as a means of evaluating N_2 fixation has been used on a wide selection of N-fixing organisms (30,31). With the use of C_2H_2 reduction to C_2H_4 , several investigators have determined the effect of various factors on N_2 fixation by nonlegume angiosperms (Alnus glutinosa (L.) Gaertn. (1,78,80), Comptonia peregrina (L.) Coult. (25), Hippophae rhamnoides L. (1,78), Myrica cerifera L. (64), and E. umbellata (45). C_2H_2 reduction by root nodules from E. umbellata has been reported (45,46).

Since no other information is available on what might influence the rate of nitrogen fixation by E. umbellata, the following study was initiated. The effect of temperature and light intensity were evaluated by use of acetylene reduction analyses. Whether there is a diurnal fluctuation in the rate of N_2 fixation was also determined.

Materials and Methods

Plant Material

Excised nodules were obtained from Elaeagnus umbellata 'Cardinal' grown in either containers or the field. One year old seedlings, 30-45 cm in height, were obtained from a midwest nursery. These were transplanted into 15 by 18 cm containers and grown for at least 6 weeks in a greenhouse at West Lafayette, IN prior to treatment. The container soil mix was peat:perlite (volume 50/50), possessing a 5.6-6.8 pH. No fertilizer was added to the growing medium

nor was additional fertilization carried out. Watering was daily with tap water.

The field grown plants were also obtained from the same Missouri nursery as seedlings and were grown an additional four seasons at West Lafayette. The growing site was a well drained sandy loam soil possessing a pH of 5.3.

Nodule Preparation

A comparison was made in order to determine if there is a difference between using excised whole nodules or just lobes when measuring reducing activity with the C_2H_2 -reduction assay.

Whole nodules from a single field-grown plant of E. umbellata 'Cardinal' were compared to excised lobes of nodules removed from the same plant. When nodule lobes were assayed, the woody basal portion of the nodule was discarded.

Soil and other debris were washed from the whole nodules or lobes without submerging them in water. Paper towels were used to remove excess water from the nodules before determining the fresh weight of each sample. The nodules were then placed in a 265 ml analysis flask which was sealed with a rubber stopple and analyzed for the reduction of C_2H_2 to C_2H_4 .

Nodules used for the remainder of the investigation were prepared as excised lobes since removal of soil and other particles was easier.

When nodules were removed from plants grown in containers, about 30 minutes elapsed between the time the nodules were excised and the time they were exposed to C_2H_2 . When nodules were removed from plants grown in the field, about 60 minutes elapsed before the nodules were exposed to C_2H_2 .

Effect of C_2H_2 Concentration

Nodules were removed from a single field-grown plant of E. umbellata 'Cardinal' and excised lobes were prepared as mentioned previously except that enough nodule material was thoroughly mixed to obtain sufficient samples for 5 treatments and 5 replications. Five C_2H_2 concentrations were evaluated on nodules once in the 17 ml flask and twice in the 265 ml flask.

For the remainder of the investigation when a 17 ml flask was used, 2 ml of air was removed with a disposable syringe prior to injecting 2 ml of commercial grade acetylene into the flask with the same syringe. When a 265 ml flask was used, 12 ml of air was removed and replaced with 12 ml of acetylene.

Analysis of C_2H_2 and C_2H_4 was completed on a Varian 1200 gas chromatograph equipped with a Porapak R column (Appendix).

Effect of Incubation Temperature

Excised lobes from nodules of field-grown E. umbellata 'Cardinal' were incubated at five temperatures (0 C, 10 C, 20 C, 30 C, 40 C) for 1 hour. Sample size was approximately 100 mg and a 17 ml analysis flask was used. The analysis flasks with nodules were submerged in water or ice baths which were maintained at the various treatment temperatures. Five replications were made at each temperature and the procedure was repeated on 3 dates within 10 days.

Effect of Light Intensity

Container grown plants of E. umbellata 'Cardinal' were grown in a greenhouse and then shifted to a growth chamber where they received 950 ft-candles or 4200 ft-candles of light for 16 hr photoperiods at 22.5 C. The plants were maintained in the growth chamber for several days prior to analysis. Analysis was using a 265 ml flask with 500 mg of excised lobes and an incubation temperature of 26 C to 28 C.

Diurnal Variation in C_2H_2 Reduction

C_2H_2 reduction analysis was made every two hours for periods of 10 to 18 hours on three dates. Lobes from nodules were prepared as described previously for container grown E. umbellata 'Cardinal'. Nodule sample size was 500 mg which was analyzed in a 265 ml flask and incubated at 26 - 28 C. Solar radiation and air temperature data were

recorded at the Purdue University National Weather Service (NWS) weather station at the Agronomy Farm, West Lafayette 6 NW. The weather station and site of container-plant production were about 8.4 km apart.

Results and Discussion

Nodule Size

When the acetylene reduction rate for whole nodules of E. umbellata 'Cardinal' was compared to sampling excised lobes from nodules, there was no significant difference between the means of the two sample materials (Table III-1). Variation between samples has been reported to be of concern by other investigators (1,25,63,78). Akkermans (1), Sloger (64), and Virtanen et al. (74) have reported reduction in C_2H_2 -reducing activity when the nodule of a nonlegume is crushed. No apparent reduction in activity was found by Silver and Mague (63) for quartered nodules. Akkermans (1) reported C_2H_2 or N_2 reduction takes place at the apical tip of the lobe. Therefore by removing an entire lobe no damage occurred that would lower the C_2H_2 -reducing rate.

Effect of C_2H_2 Concentration

As the concentration of C_2H_2 increased to approximately 10%, there was an increase in the amount of C_2H_4 produced when using the 17 ml analysis flask (Figure III-1). Above 10% C_2H_2 there was a decrease in C_2H_4 produced. Mague

Table III-1. The effect of whole or divided nodules on sample variation.

Nodule material	Samples				Mean ^Z	Standard error of mean
	1	2	3	4	5	
	$\mu\text{l C}_2\text{H}_4/\text{g nodule/hr}$					
whole nodules	22.0	236.2	188.6	16.9	277.0	148.1 a
						54.4
excised lobes from nodules	84.4	277.1	161.0	112.8	138.6	154.8 a
						33.2

^ZMeans were not significantly different at 5% level.

Figure III-1. The effect of C_2H_2 concentration on C_2H_2 reduction to C_2H_4 by nodules from E. umbellata 'Cardinal' when using a 17 ml analysis flask. The points are means of 10 values obtained from 5 samples analyzed on each of 2 days.

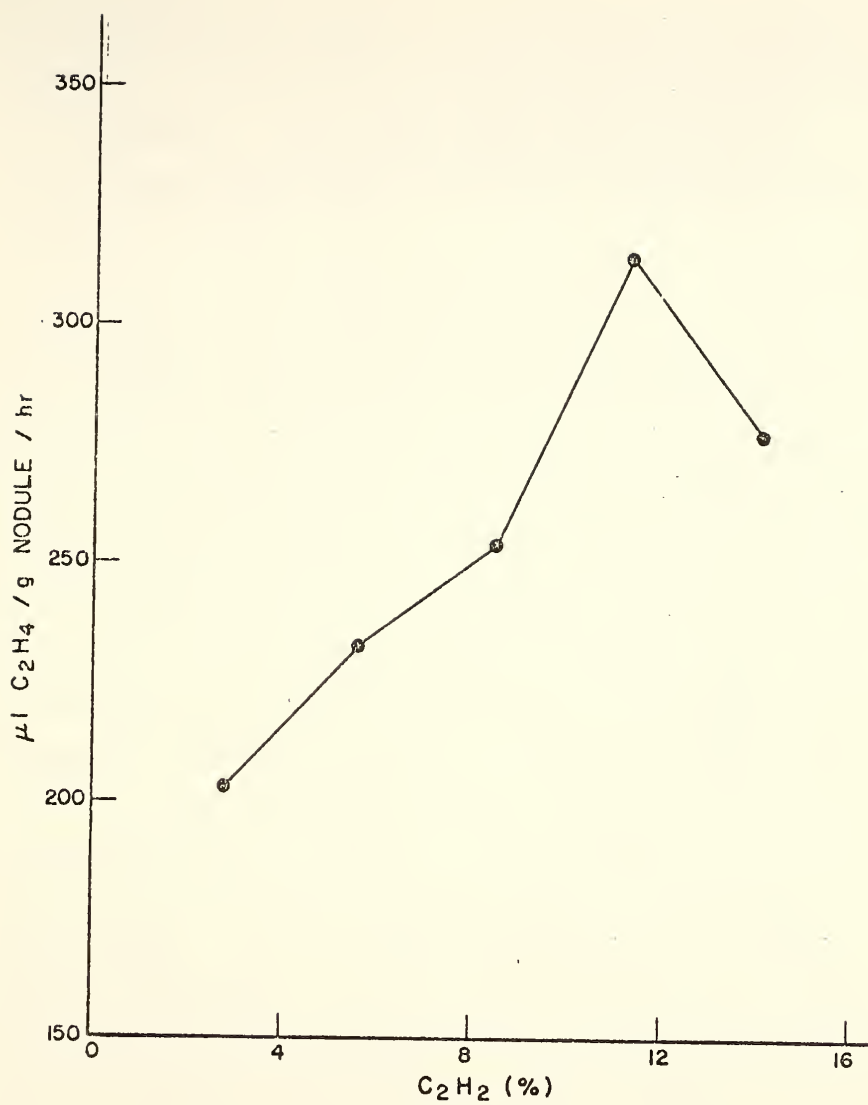


Figure III-1.

and Burris (43) attribute a similar reduction in C_2H_4 production to the alteration of other gases as C_2H_2 concentration increases. When using a 265 ml flask the rate of C_2H_4 production was still increasing at 9.1% C_2H_2 (Figure III-2).

Effect of Temperature

Excised lobes of nodules from E. umbellata 'Cardinal' had maximum C_2H_2 reduction at approximately 30 C (Figure III-3). Reducing activity was minimal at 0 C, increased rapidly with temperature increases through 30 C, and declined in C_2H_4 production above 30 C. McNiel and Carpenter (47) had also reported 30 C as the temperature for maximum reduction for several other nonlegumes. Fessenden et al. (25) reported maximum C_2H_2 reduction at 30 C for nodules from Comptonia peregrina (L.) Coult. Wheeler (80) had found 25 C as the temperature for maximum reducing activity for Alnus glutinosa (L.) Gaertn. The temperature for maximum reducing activity has also been reported to be 26 C for Alnus viridis DC. (9), Alnus glutinosa (1,78), and Hippophae rhamnoides (L.) (1,78). When the data for E. umbellata 'Cardinal' is transformed into an Arrhenius plot, the plot is biphasic (Figure III-3). This indicates that there is a change in the activation energy for nitrogenase between 10 C and 20 C. The change occurs at approximately 14 C.

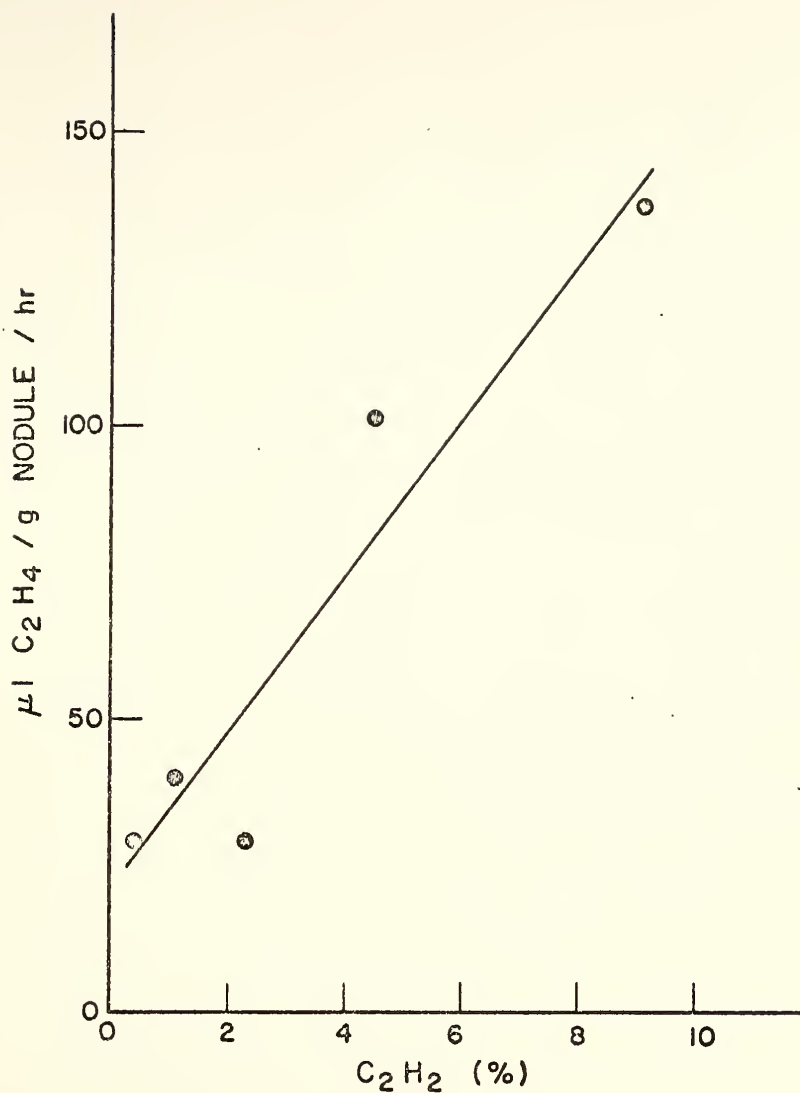


Figure III-2. The effect of C_2H_2 concentration on C_2H_2 reduction to C_2H_4 by nodules from E. umbellata 'Cardinal' when using a 265 ml analysis flask.

Figure III-3. Effect of temperature on the reduction of C_2H_4 using excised lobes of nodules from E. umbellata 'Cardinal'. Points are the means of 15 samples, 5 samples on each of 3 days. The vertical bars are $\pm$ the standard error of the mean. The insert is the Arrhenius plot of the same data.

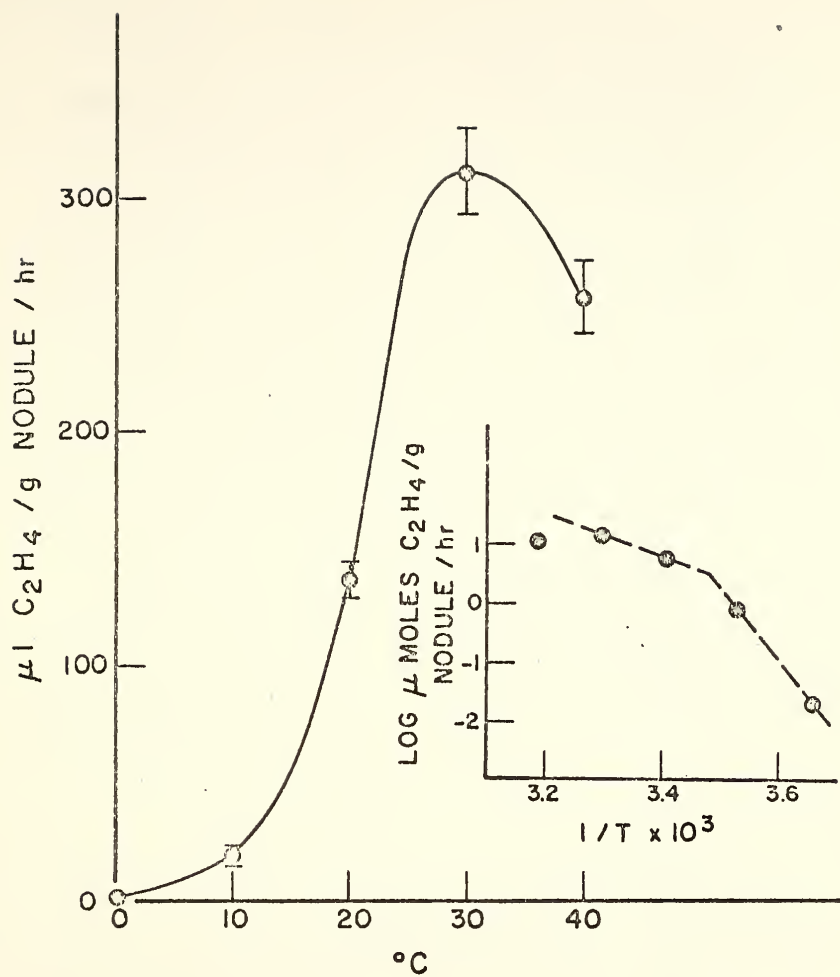


Figure III-3.

Effect of Light Intensity

No significant difference occurred when plants of E. umbellata 'Cardinal' were treated at 950 ft-candles or at 4200 ft-candles (Figure III-4). Differences did occur from day to day at both intensities that can not be explained. Under field conditions, changes in light intensity above 950 ft-candles apparently will have no effect on the reducing activity of nodules. Wheeler and Bowes (81) had found A. glutinosa to respond with increased C_2H_2 reduction up to 864 ft-candles. They had also proposed additional increases at higher light intensities.

Diurnal Variation in C_2H_2 Reduction

Diurnal variation in C_2H_2 -reducing activity as had been reported for other crops (31,43,79,80) was not confirmed by these studies. As illustrated in Figures III-5 through III-7, on days of high light intensity, the lowest rates of C_2H_2 reduction did not occur during the hours prior to sunrise as had been reported for Glycine max Merr. (31,43). For G. max, Pisum sativum (L.), and A. glutinosa; light, air temperature and photosynthates have been correlated to changes in the C_2H_2 -reducing ability of nodules (40,43,80). The highest light intensity and photosynthate translocation has been reported at midday, as well as the highest C_2H_2 -reducing rate (43,79,80).

The 20 June 1974 sampling occurred during a period of cloudy and rainy weather (Figures III-5 and IV-5). No large

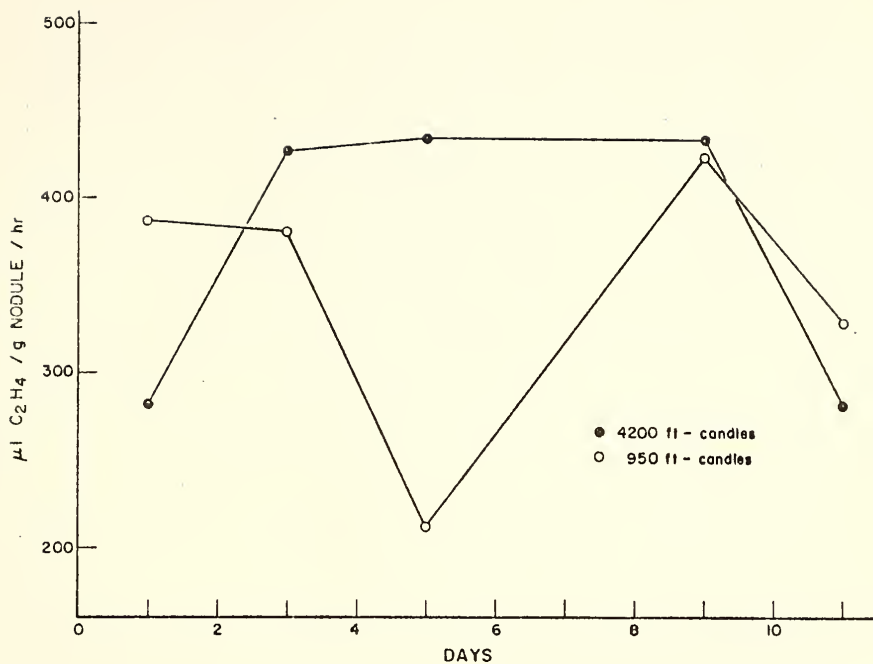


Figure III-4. The effect of 2 light intensities (950 and 4200 ft-candles) on the rate of C_2H_2 reduction to C_2H_4 by excised nodules from *E. umbellata* 'Cardinal'.

Figure III-5. A comparison of the diurnal variation in the reduction of C_2H_2 to C_2H_4 by excised nodules collected from E. umbellata 'Cardinal' on 20 June 1974 to solar radiation and air temperature. Each point for C_2H_4 is a mean of 5 samples, each collected from a different plant. The vertical bars are $\pm$ the standard error of the mean.

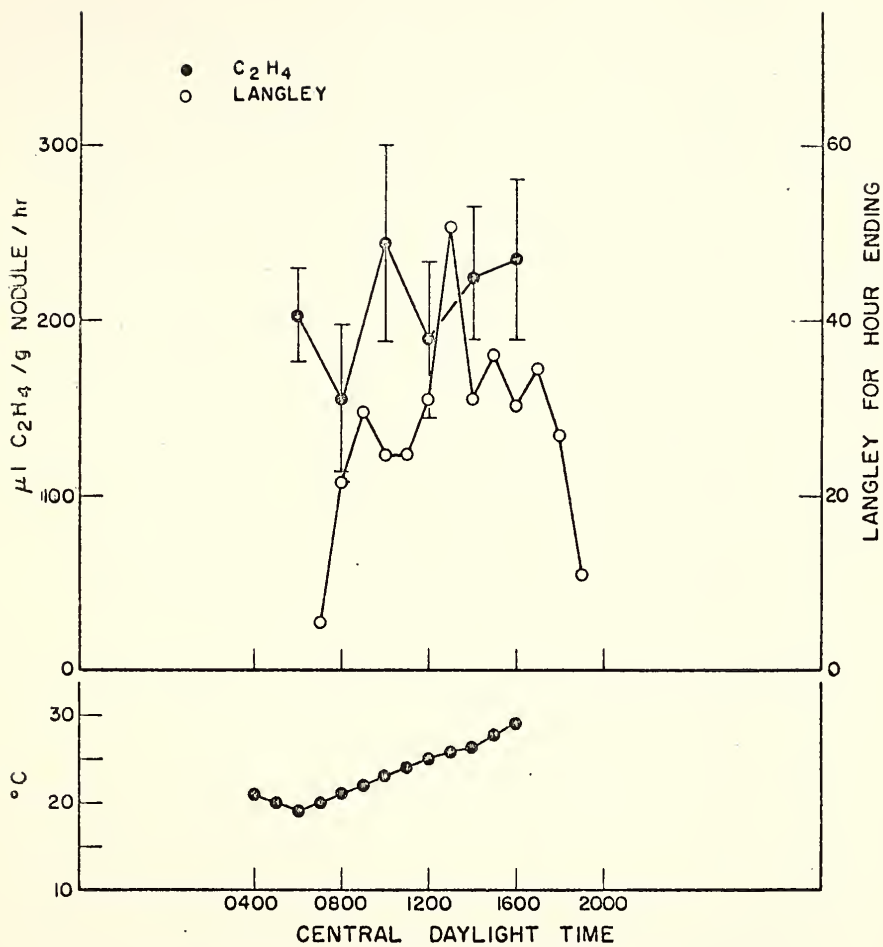


Figure III-5.

Figure III-6. A comparison of the diurnal variation in the reduction of C_2H_2 to C_2H_4 by excised nodules collected from E. umbellata 'Cardinal' on 26 June 1974 to solar radiation and air temperature. Each point for C_2H_4 is a mean of 5 samples, each taken from a different plant. The vertical bars are $\pm$ the standard error of the mean.

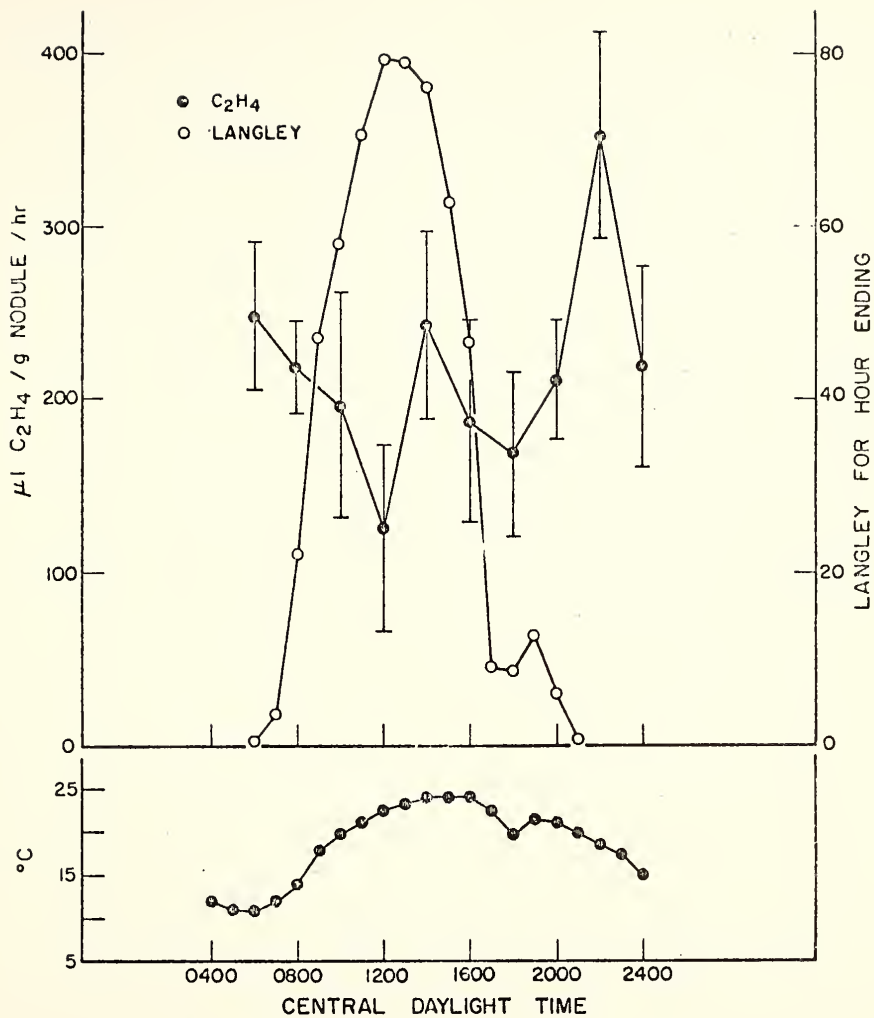


Figure III-6.

Figure III-7. A comparison of the diurnal variation in the reduction of C_2H_2 to C_2H_4 by excised nodules collected from E. umbellata 'Cardinal' on 16 July 1974 to solar radiation and air temperature. Each point for C_2H_4 is a mean of 5 samples, each taken from a different plant. The vertical bars are $\pm$ the standard error of the mean.

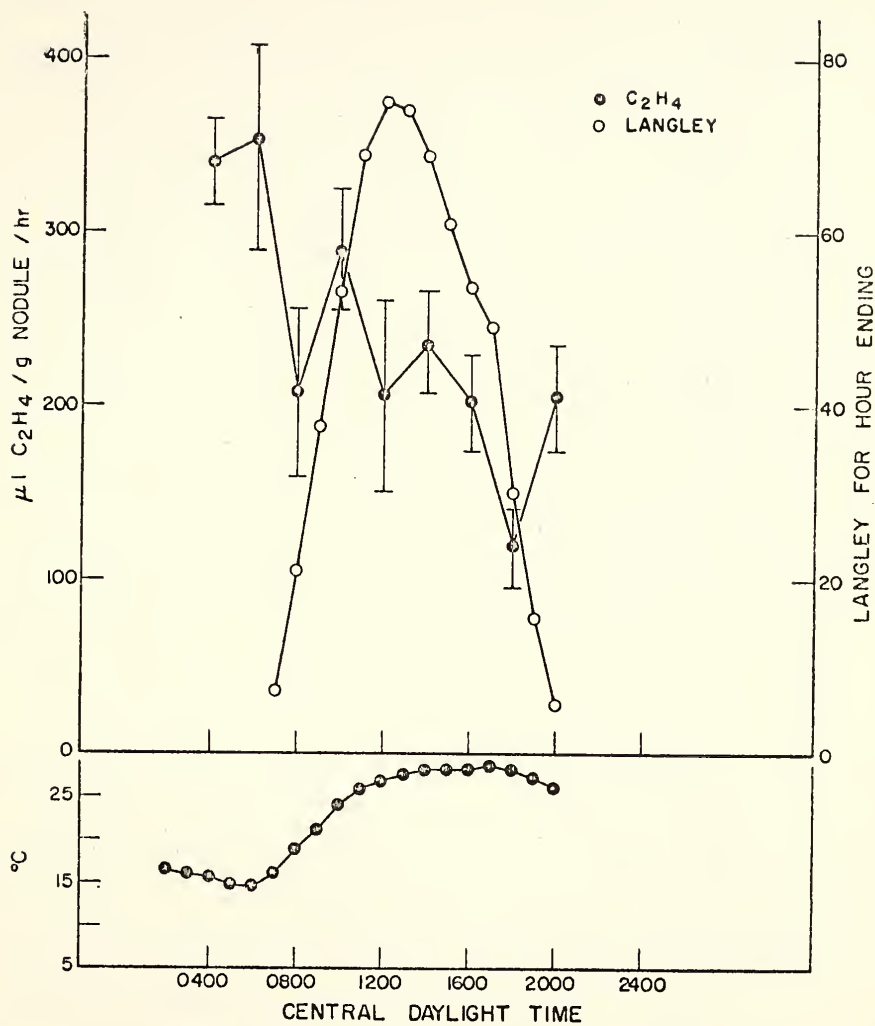


Figure III-7.

variation occurred in the amount of C_2H_4 produced from nodules between 0600 and 1600 hours. A slight increase in the mean values of C_2H_4 produced did occur as the day progressed. This increase corresponds to the increase in solar radiation and temperature as the day progressed.

Sampling on 26 June 1974 occurred on a bright clear day that was interrupted by cloudiness (Figure III-6) and rain (Figure IV-5) after 1500 hours. The mean values of C_2H_4 produced decreased prior to 1200 hours. This trend was reversed between 1200 and 2400 hours (Figure III-6). The slight increase in C_2H_4 produced between 1200 and 2400 hours may be due to an increase in carbohydrate production followed by a lag period until the carbohydrate reached the root system. The fluctuation during this 12 hour period does not relate to changes in light intensity or temperature.

The 16 July 1974 sampling occurred on a bright sunny day (Figure III-7) which had not been preceded by dense clouds or rain for several days. Ethylene produced by nodules during predawn hours was at as high a level as occurred on any of the three sampling dates. During the day the reducing activity declined until reaching the lowest level near the end of the light period.

There definitely was no increase in activity due to an increase in solar radiation.

Dale and Hodges (19) have warned against using only solar radiation as an estimate for corn yield (photosynthate production). Their reasoning is that there is an interaction between solar radiation and water stress. Sprent and Engin and Sprent (23,67,68) have reported reductions in nitrogen-fixing activity to be the result of moisture stress.

Believing that high light intensity, low soil volume and thus low water volume, since the plants were containerized, could lead to water stress, the factors affecting water stress were evaluated. Linvill (42) has proposed a moisture stress weighting factor for corn that consisted of solar radiation over evaporation rate. When applied to E. umbellata on an hour to hour basis using data obtained from weather station: West Lafayette, 6 NW (unpublished, provided by R.F. Dale), no positive relationships could be established between the moisture stress factor and C_2H_4 produced during the day. However, moisture stress is still believed to be a factor that has affected C_2H_2 reduction. The evaporation value from a standard class A evaporation pan published by weather station: West Lafayette, 6 NW (17) was the highest (11.9 mm) on 16 July 1974 for any date during the period 20 June to 1 August 1974. The lowest value (1.5 mm) occurred on 24 July. Values for 20 June and 26 June were 3.81 mm and 6.35 mm respectively. Thus no reduction in the C_2H_4 production rate occurred when solar radiation and evaporation were low (20 June 1974 -

Figure III-5) but reduction was great during the day when solar radiation and evaporation were high (16 July 1974 - Figure III-7).

Conclusions

Excised lobes from nodules obtained from E. umbellata 'Cardinal' have the ability to reduce C_2H_2 at rates similar to those produced by excised whole nodules. The rate of C_2H_2 reduced by excised nodules is influenced by temperature. The maximum rate for C_2H_4 produced occurs at 30 C. The rate increases rapidly from 0 C to 30 C and declines above 30 C. There appears to be no increase in the rate of C_2H_4 produced with an increase in the light intensity from 950 to 4200 ft-candles.

No increase in C_2H_4 produced occurred with increased solar radiation during the day. Fluctuation did occur that was attributed to moisture stress. With moisture stress the rate of C_2H_4 produced decreased. Stress was greatest on a day with high solar radiation and high evaporation while low on a day with low solar radiation and low evaporation.

CHAPTER IV

THE EFFECT OF TEMPERATURE, SOLAR RADIATION, AND
MOISTURE ON THE REDUCTION OF ACETYLENE
BY EXCISED ROOT NODULES FROM
ALNUS GLUTINOSA

Introduction

Since Stewart et al. (70) reported a difference in reducing activity between samples taken in the morning and samples taken in the afternoon, various factors have been related to the N_2 -fixing capabilities of root nodules. Wheeler and Fessenden et al. have related fluctuations in C_2H_2 -reducing activity to the time of day (25,79) and to temperature (25,80). Sprent (67) added soil moisture while Mague and Burris (43) added light intensity and season to time of day and temperature as the factors evaluated in order to determine reasons for daily variation in reducing activity.

Wheeler (79) and Mague and Burris (43) both found maximum C_2H_2 reduction by nodules to occur from midday through early afternoon. Wheeler held temperature constant and was then able to relate the increase in C_2H_4 produced by nodules from Alnus glutinosa (L.) Gaertn. to high light intensity during the sampling period. Field grown Glycine

max Merr. (soybean) were sampled by Mague and Burris and the fluctuation in C_2H_4 produced was related to air temperature and not specifically to light intensity.

Temperature influences on C_2H_2 reduction have been characterized for several species (1,9,25,47,78,80). There is a definite increase in reducing activity as the temperature increases from 5 C to 20-30 C.

Fessenden et al. (25) was not able to show a diurnal fluctuation using nodules from Comptonia peregrina (L.) Coult. However, the temperature or light intensity for the sampling period was not reported. In sampling Arachis hypogea L. (peanut), Balandreau et al. (6) found a definite decrease in C_2H_2 -reducing activity during midday. Though not measured, this reduction was believed to be a response to low atmospheric and soil moisture. The assumption made by Balandreau et al. (6) may be valid since Sprent and Engin and Sprent (23,67,68) have found lower values for acetylene reduction when various legumes are under moisture stress.

The following study was conducted in order to further characterize the influence of light, temperature and moisture on N_2 -fixing ability of A. glutinosa.

Materials and Methods

Plant Material

Nodules used for analysis were removed from plants of A. glutinosa grown from seed in a greenhouse in West Lafayette, IN. Seedlings were obtained from three different sowings in 1971, 1972, and 1974. All seedlings were transplanted into 12 cm x 12 cm plastic containers for additional development. When large enough, seedlings from the 1971 and 1972 sowings were transplanted into the field. The container media was peat:perlite (50:50 by volume) which possessed a 5.6 pH. The field site was a sandy loam with a 6.5 pH. No additional fertilization was made to any of the plants. The container stock was watered daily with tap water and the field stock in its third growing season in 1974 was irrigated periodically.

Nodule Preparation

Nodules were divided and only the lobes assayed. The basal woody portion of each nodule was discarded. Soil and debris was removed from the lobes with tap water. Nodules were then blotted dry on paper towels. Fresh weights were determined before placing the nodules in an analysis flask.

Approximately 30 minutes elapsed between excising the nodule and placing it in the analysis flask. When nodules were removed from field grown plants, preparation took about 60 minutes.

Measurement of Acetylene Reduction

The acetylene reduction assay was used to measure the N_2 -fixing ability of the nodules. Approximately 1 g of nodule tissue was placed in a 265 ml analysis flask and closed with a rubber stopple. With the use of a 12 ml disposable syringe, 12 ml of air was removed from the flask and replaced with 12 ml of commercial grade C_2H_2 . After an incubation time of 1 hour at 26-28 C, a 1 ml sample was removed from the flask and analyzed on a Varian 1200 gas chromatograph equipped with a Porapak R column (Appendix).

Effect of Light Intensity

Approximately 7 month old container grown A. glutinosa, 20 cm in height, were removed from a greenhouse environment and placed in growth chambers for the light treatments a few days prior to acetylene analysis. The two light intensities used were 950 ft-candles and 4200 ft-candles at foliage height. Watering was daily and the temperature was 22.5 C. Nodules were removed 1 to 3 hours after the beginning of a 16 hour photoperiod.

Diurnal Fluctuation in the Rate of

Acetylene Reduction

Excised nodules removed from 3 year old field grown A. glutinosa were analyzed for variation in the production of C_2H_4 from C_2H_2 at two hour intervals on three different dates. Destructive sampling was used with samples taken

from each of 5 individual plants at each time interval. Nodule preparation and analysis was carried out as described previously.

Climatological data reported by the Purdue University-National Weather Service (NWS) weather station at the Agronomy Farm, West Lafayette 6 NW (17) was used (solar radiation data is unpublished, but provided by W.L. Stirn). The weather station is located 8.4 km from the growing site.

The Day by Day Variation in the Rate of Acetylene Reduction

Nodules from 4 year old field grown A. glutinosa were sampled at 1200 hours on several days during a 41 day period. On each day destructive sampling was used to obtain samples from each of 5 individual plants. Nodule preparation and analysis of C_2H_4 production from C_2H_2 was carried out as mentioned previously. Precipitation data reported by the weather station at West Lafayette FAA AP was used (17). The weather station was located 1 km from the growing site.

Results and Discussion

Effect of Light Intensity

Acetylene-reduction activity by excised nodules obtained from A. glutinosa did not respond to an increase in light intensity from 950 to 4200 ft-candles (Figure IV-1). These findings agree with those found for E. umbellata (48) but disagree with what was proposed by Wheeler and Bowes

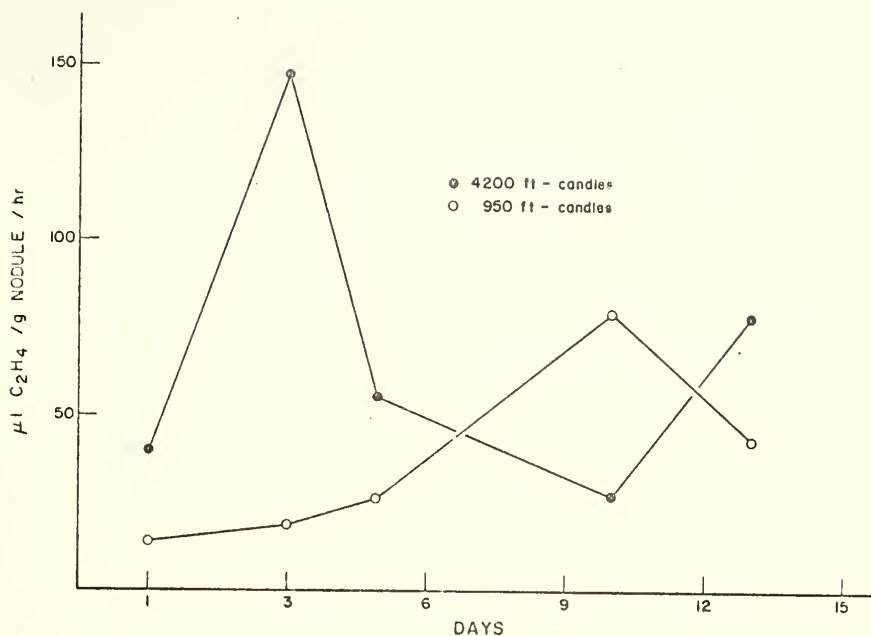


Figure IV-1. The effect of 2 light intensities (950 and 4200 ft-candles) on the rate of C_2H_2 reduction to C_2H_4 by excised nodules from A. glutinosa. Each point is the mean of 5 replications.

(81). The rate of C_2H_4 produced did tend to increase between the 1st and 11th day for plants grown under both light intensities, thus indicating a response to age. This response may be due to the young age of the plants.

Diurnal Fluctuation in the Rate of Acetylene Reduction

Fluctuations in C_2H_2 -reducing activity occurred approximately one hour after fluctuations occurred in solar radiation for samples taken on 20 June 1974 from nodules on field grown A. glutinosa (Figure IV-2). With an increase in solar radiation between 0700 and 0900 hours, corresponding increases occurred in the quantity of C_2H_4 produced by samples analyzed at 1000 hours versus 0800 hours. Similar C_2H_4 increases were observed when solar radiation increased between 1100 and 1300. Decreases in C_2H_4 produced between 1000 and 1200 hours and between 1400 and 1600 hours corresponded to decreases in solar radiation due to variable cloudiness between 0900 and 1100 hours and between 1300 and 1500 hours respectively. The overall increase in C_2H_2 -reducing activity between 0800 and 1600 hours corresponded to a similar increase in both solar radiation and temperature during this period. The variation between 0800 and 1600 hours is similar to the results Mague and Burris found using field grown soybeans (43). They attributed the increase in reducing activity to an increase in temperature.

Due to variation that was not representative of the other samples, 9 samples (i.e., 8 samples had essentially

Figure IV-2. A comparison of the diurnal variation in the reduction of C_2H_2 to C_2H_4 by excised nodules collected from A. glutinosa on 20 June 1974 to solar radiation and air temperature. Each point for C_2H_4 is a mean of 5 samples, each taken from a different plant. The vertical bars are $\pm$ the standard error of the mean.

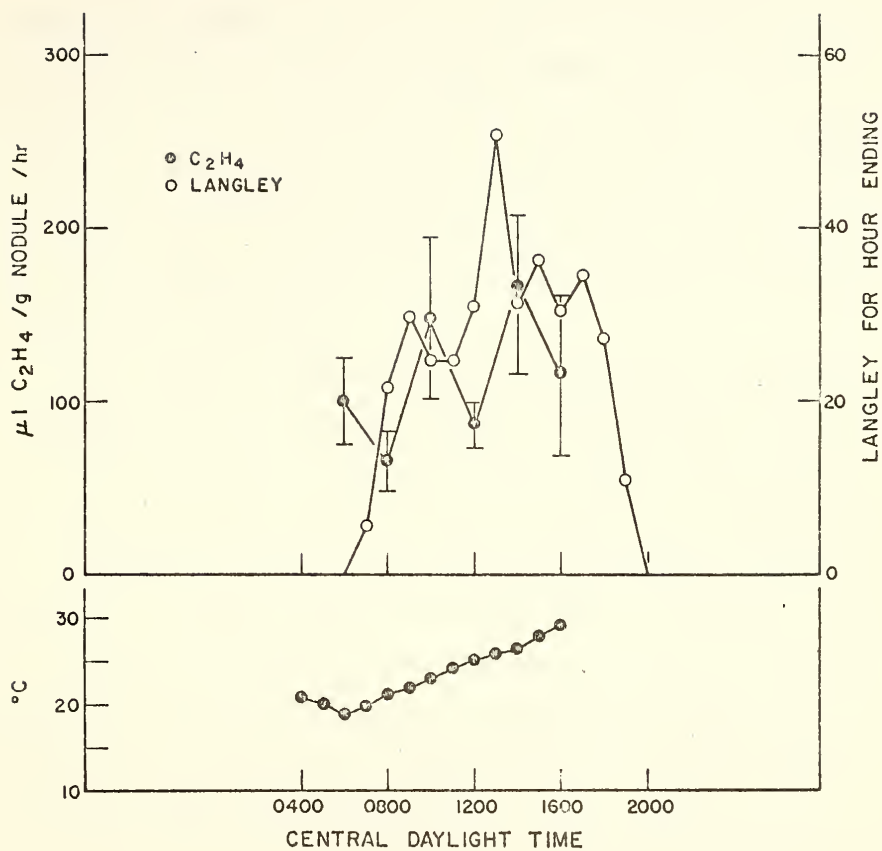


Figure IV-2.

no activity, while 1 sample was unusually high) of the 90 analyzed were removed from the data reported for 26 June 1974 and 16 July 1974.

On 26 June 1974 when full sunlight increased the solar radiation received over that received on the cloudy day of 20 June 1974, the levels of C_2H_4 produced were higher (Figure IV-3). As on 20 June 1974, the trend was for C_2H_4 production to increase or decrease as did solar radiation and temperature between 0800 and 1800 hours. Cloudiness and rain caused the decrease in solar radiation and temperature after 1500 hours.

The rates of C_2H_4 produced during the dark periods (around 0600 hours and between 2000 and 2400 hours) were not at the lowest levels for the day. These higher rates do not correspond to what has been reported or proposed with A. glutinosa or other crops (31,43,79,80,81). The size and age of the various species investigated are different. The A. glutinosa in this study had reached 3 to 4 m in height during the 4th growing season. Carbohydrate reserves in this larger plant may be responsible for the higher reducing rate obtained from nodules.

The analysis on samples obtained on 16 July 1974 found the rate of C_2H_4 produced to be low compared to 26 June 1974 even though solar radiation and temperature were similar on the two dates (Figures IV-3 and IV-4). For 16 July 1974, there was no response in C_2H_2 -reducing

Figure IV-3. A comparison of the diurnal variation in the reduction of C_2H_2 to C_2H_4 by excised nodules collected from A. glutinosa on 26 June 1974 to solar radiation and air temperature. Each point for C_2H_4 is a mean of 5 samples, each taken from a different plant. The vertical bars are $\pm$ the standard error of the mean.

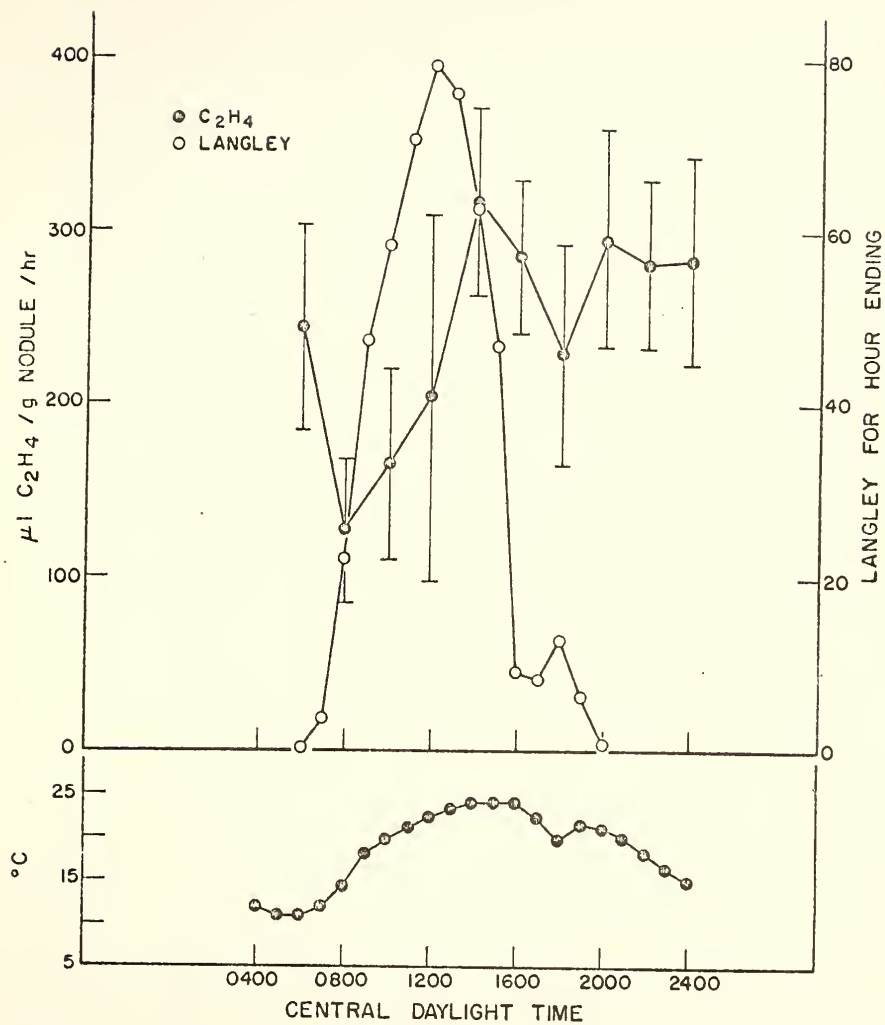


Figure IV-3.

Figure IV-4. A comparison of the diurnal variation in the reduction of C_2H_2 to C_2H_4 by excised nodules collected from A. glutinosa on 16 July 1974 to solar radiation and air temperature. Each point for C_2H_4 is a mean of 5 samples, each taken from a different plant. The vertical bars are $\pm$ the standard error of the mean.

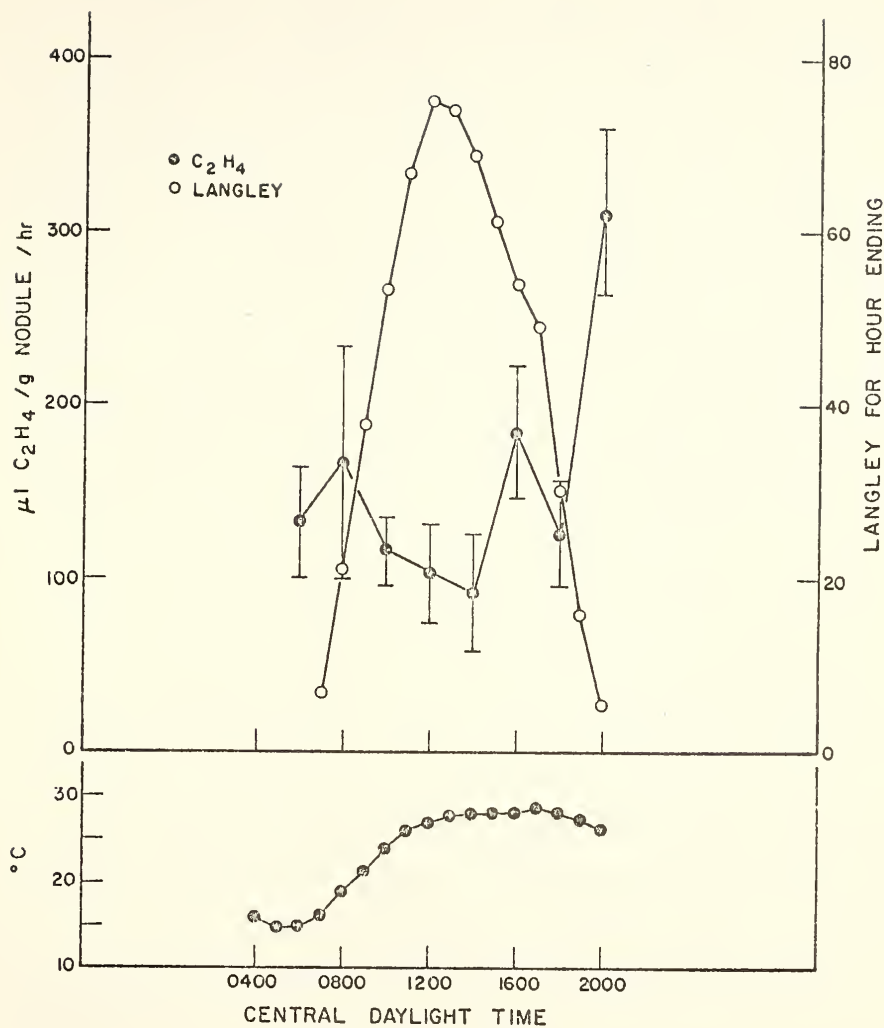


Figure IV-4.

activity that corresponded to increase in solar radiation or temperature (Figure IV-4).

The lack of response is believed to be due to moisture stress. Engin and Sprent as well as Sprent (23,67,68) have determined that C_2H_2 reduction by nodules is reduced when plants are under moisture stress. No natural precipitation had occurred by this date in July. The evaporation rate (11.9 mm/day) reported by weather station: West Lafayette 6 NW for 16 July 1974 was the highest recorded between 1 June and 1 August 1974 (17). Even though the A. glutinosa sampled had been irrigated on occasion, the lack of water from any other source and high evaporation rates still may have caused water stress to these plants.

The Day by Day Variation in the Rate of Acetylene Reduction

Variations occurred in the C_2H_4 produced by nodules excised from plants during the 41 day period (Figure IV-5). The highest rates of C_2H_4 production occurred on 25 June, 1 July, and 25 July and each of these dates was preceded by a period of rainfall. Nodules remained active during a 22 day period of no precipitation. However, the levels of C_2H_4 produced were below the levels produced when water was available.

The low level of activity during the period 15 July to 17 July 1974 may confirm the lack of response to solar

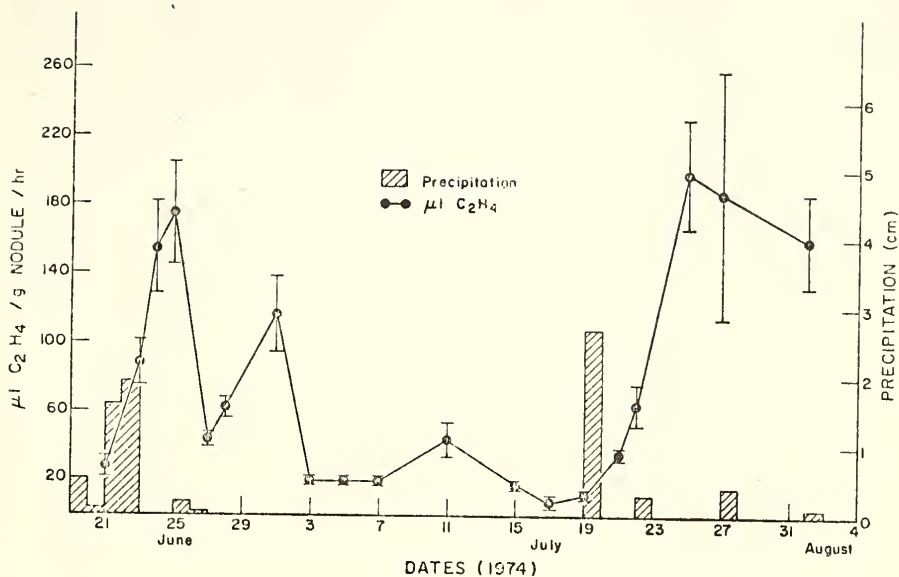


Figure IV-5. The influence of moisture on C_2H_2 reduction to C_2H_4 by nodules obtained from *Alnus glutinosa* over a 41 day period. Each point for C_2H_4 is the mean of 5 samples, each taken from a different plant. The vertical bars are $\pm$ the standard error of the mean.

radiation and temperature reported in the previous section as having occurred on 16 July 1974.

Conclusions

An increase in light intensity from 950 to 4200 ft-candles around plants grown in a growth chamber did not produce a change in the rate of acetylene reduction. When field grown plants have ample moisture, they do respond to fluctuations in solar radiation, temperature or both. With increases in solar radiation and temperature, increases occurred in the quantity of C_2H_2 reduced to C_2H_4 by excised nodules. When moisture was limiting, there was no response in C_2H_4 produced to solar radiation or temperature.

Over time, nodules appear to fix nitrogen at high rates only when ample soil moisture is available.

CHAPTER V
THE EFFECT OF SURFACE DISINFECTANTS ON C_2H_2 REDUCTION
BY NODULES REMOVED FROM ALNUS GLUTINOSA

Introduction

Surface disinfectants are used to disinfect nodules prior to placing nodules on a culture medium. Loss of endophyte virulence when nodules are placed on a culture medium has been reported (58). Part of the loss was considered to be due to the effect of the disinfecting solution. The effect of several disinfecting materials on N_2 -fixing activity by nodules of Alnus glutinosa was studied.

Materials and Methods

Nodules were obtained from field-grown A. glutinosa and prepared and analyzed for C_2H_2 reduction as has been reported previously in Chapter IV. Once the nodule lobes were cleaned, they were treated for either 1 or 10 minutes in solutions of 0.525% $NaClO_4$, 1% $AgNO_3$, tap H_2O , or de-ionized H_2O (Table V-1) prior to exposure to C_2H_2 .

Results and Discussion

The use of 1% $AgNO_3$, 0.525% $NaClO_4$, and H_2O decreased the amount of C_2H_2 reduced compared to that reduced if not treated (Table V-1). The use of 1% $AgNO_3$ when used for

Table V-1. A comparison of C_2H_4 production by excised nodules treated with various surface disinfectants for 1 or 10 minutes.

Treatment	Time	Days		
		1	2	3
	(min)	$\mu l C_2H_4/g \text{ nodule/hr}$		
Air		128.5		21.7
Tap H_2O	10			15.0
Deionized H_2O	10			13.6
0.525% Sodium Hypo- chlorite	1			16.4
0.525% Sodium Hypo- chlorite	10	45.4	50.8	10.5
1% $AgNO_3$	1		2.0	6.6
1% $AgNO_3$	10	3.7	5.4	

either 1 or 10 minutes had the greatest influence on decreasing the reducing activity. Loss of the endophyte may definitely be the result of use of disinfecting solutions. Because of the loss in reducing activity, the type of disinfectant and the duration of its use should be considered before treating nodules that are to be placed on a culture medium.

CHAPTER VI

C_2H_2 GAS STANDARDS

Introduction

When these studies were initiated, an available source of C_2H_4 had a known concentration of 0.913 ppm C_2H_4 . From the sample peaks formed on the gas chromatograph printout from the C_2H_2 reduction by nodules, the C_2H_4 levels were too great to permit the use of the 0.913 ppm C_2H_4 source as a standard.

A source of reportedly 'pure' C_2H_4 was used as a standard comparison against the sample peaks obtained from C_2H_4 sources to determine an accurate value for the 'pure' C_2H_4 .

Materials and Methods

A dilution of the 'pure' C_2H_4 was made with a gas-tight 100 μ l syringe by placing 100 μ l 'pure' C_2H_4 into a stoppered 265 ml flask (Flask A) containing a few polystyrene beads. To insure mixing, all treatment flasks were shook briefly. A 1 ml sample was removed from flask A with a syringe and placed in flask B which also contained a few polystyrene beads. A 1 ml sample was removed from flask B and injected into a gas chromatograph. The resulting peak

produced by the gas chromatograph was compared to a similar peak produced from a 1 ml sample from the 0.913 ppm C_2H_4 source. This procedure was replicated 5 times.

Results and Discussion

The results of the comparison between the C_2H_4 sources are presented in Table VI-1. The mean value for all computed values, if the gas in the second tank was pure, was 1.400 ppm C_2H_4 compared to a mean value of 1.590 for all samples measured on the gas chromatograph. The gas in the second tank was considered pure C_2H_4 even with the slight variation in values. A volume variation of $\pm 1\%$ with the 100 μ l syringe and the lack of being able to have precise measurements of flasks A and B in μ l may have influenced the variation between the measured and computed means.

Using the gas in flask B of sample 1 (Table VI-1) a standard curve was formed from varying sample sizes (Figure VI-1). During analysis on nodules, standard curves were established for several ranges of concentrations of C_2H_4 . These were repeated as necessary.

With the use of the regression program SPSS 100, Version 5.0, 15 Dec. 1972, a computer program developed a formula for a straight line using the values obtained from the standard curve. This formula was then used to determine the μ l C_2H_4 produced by an unknown sample by using the peak height response from the sample.

Table VI-1. The determination of an unknown concentration of C_2H_4 from a known standard of 0.913 ppm C_2H_4 .

Sample No.	Volume ^z flask A	Volume ^z flask B	Compared to ^y standard using a gas chromatograph	Computed from the volume
	ml	ml	ppm C_2H_4	ppm C_2H_4
1	264.9	266.1	1.511	1.418
2	266.7	270.3	1.522	1.382
3	269.5	267.6	1.560	1.387
4	267.1	265.6	1.571	1.410
5	265.7	267.9	1.788	1.405

^zMeans of 5 measurements.

^yMeans of 3 samples.

Figure VI-1. A standard curve prepared for ethylene (C_2H_4) using a Varian 1200 gas chromatograph. Samples were taken from a 266.1 ml flask containing 0.3775 μ l C_2H_4 and peaks were compared to the peak of a 1 ml sample of gas containing 0.913 ppm C_2H_4 .

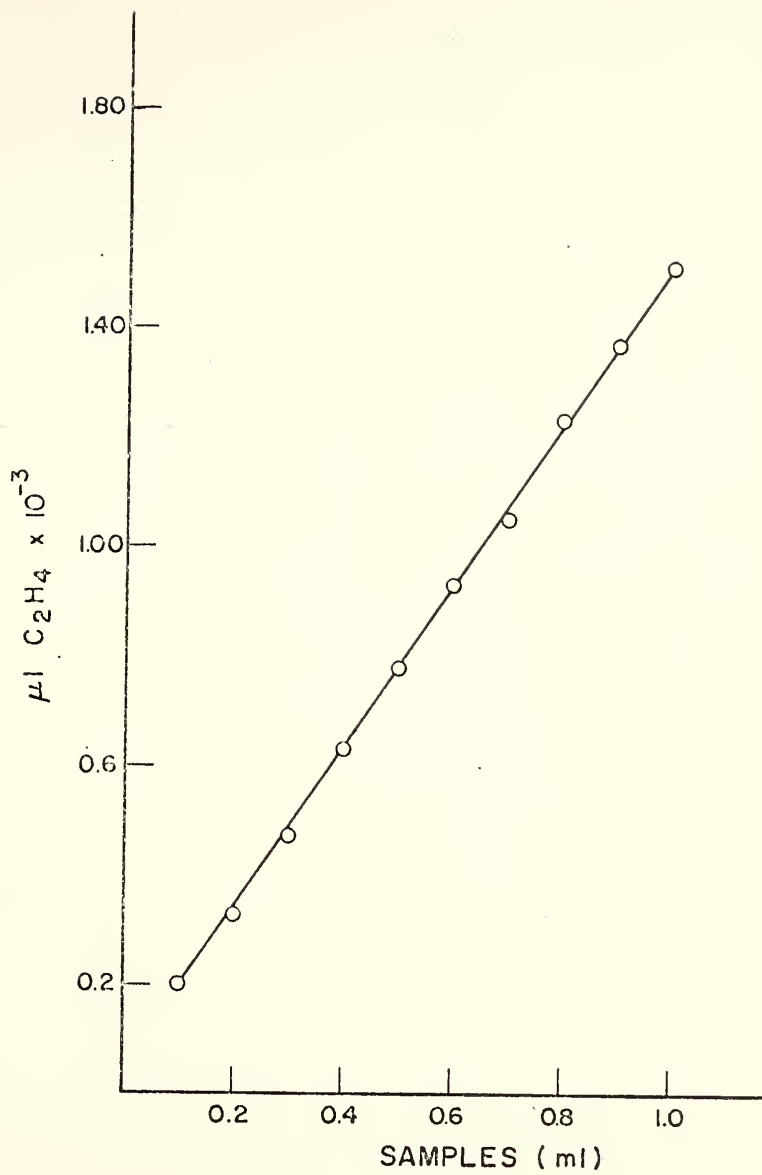


Figure VI-1.

LITERATURE CITED

LITERATURE CITED

1. Akkermans, A.D.L. 1971. Nitrogen fixation and nodulation of *Alnus* and *Hippophae* under natural conditions. Ph.D. Thesis, University of Leiden, The Netherlands.
2. Allan, P.F. and W.W. Steiner. 1965. Autumn Olive for wildlife and other conservation uses. U.S. Dept. Agric. Leaflet No. 458.
3. Allen, E.K. and O.N. Allen. 1965. Nonleguminous plant symbiosis. In: Microbiology and Soil Fertility. C.M. Gilmour and O.N. Allen, eds. Oregon State Univ. Press, Corvallis. 77-106.
4. Angulo Carmona, A.F. 1974. La formation des nodules fixateurs d'azote chez *Alnus glutinosa* (L.) Vill. Acta Bot. Neerl. 23:257-303.
5. Aprison, M.H., W.E. Magee, and R.H. Burris. 1954. Nitrogen fixation by excised soybean root nodules. J. Biol. Chem. 208:29-39.
6. Balandreau, J.P., C.R. Millier, and Y.R. Dommergues. 1974. Diurnal variations of nitrogenase activity in the field. App. Microbiol. 27:662-665.
7. Becking, J.H. 1970. Frankiaceae FAM. NOV. (Actinomycetales) with one new combination and six new species of the genus *Frankia* BRUNCHORST 1886, 174. Int'l. J. Syst. Bact. 20:201-220.
8. Becking, J.H. 1970. Plant-endophyte symbiosis in non-leguminous plants. Plant Soil 32:611-654.
9. Benecke, U. 1970. Nitrogen fixation by *Alnus viridis* (Chaix) DC. Plant Soil 33:30-48.
10. Bergersen, F.J. 1970. The quantitative relationship between nitrogen fixation and the acetylene-reduction assay. Aust. J. Biol. Sci. 23:1015-1025.
11. Bergersen, F.J. 1971. Biochemistry of symbiotic nitrogen fixation in legumes. Ann. Rev. Plt. Physiol. 22:121-140.

12. Bond, G. 1955. An isotopic study of the fixation of nitrogen associated with nodulated plants of Alnus, Myrica, and Hippophae. J. Exp. Bot. 6:303-311.
13. Bond, G. 1957. Isotopic studies of nitrogen fixation in non-legume root nodules. Ann. Bot. 21:513-521.
14. Bond, G. 1963. The root nodules of non-leguminous angiosperms. Symp. Soc. Gen. Microbiol. 13:72-91.
15. Bond, G. 1967. Fixation of nitrogen by higher plants other than legumes. Ann. Rev. Plt. Physiol. 18:107-126.
16. Bond, G., W.W. Fletcher, and T.P. Ferguson. 1954. The development and function of the root nodules of Alnus, Myrica, and Hippophae. Plant Soil 5:309-323.
17. Climalogical Data. 1974. Indiana station: West Lafayette FAA AP. U.S. Dept. Commerce, Environmental Data Service, Ashville, N.C. Vol. 79.
18. Crocker, R.L. and J. Major. 1955. Soil development in relation to vegetation and surface age at Glacier Bay, Alaska. J. Ecol. 43:427-448.
19. Dale, R.F. and H.F. Hodges. 1975. Weather and Corn Yield Study. Final Report, Environ. Data Ser., Nat'l Oceanic and Atmos. Admin., Washington, D.C.
20. Dalton, D.A. and A.W. Naylor. 1975. Studies on nitrogen fixation by Alnus crispa. Amer. J. Bot. 62:76-80.
21. Delwiche, C.C., P.J. Zinke, and C.M. Johnson. 1965. Nitrogen fixation by Ceanothus. Plt. Physiol. 40: 1045-1047.
22. Dilworth, M.J. 1966. Acetylene reduction by nitrogen-fixing preparations from Clostridium pasteurianum. Biochim. Biophys. Acta 127:285-294.
23. Engin, M. and J.I. Sprent. 1973. Effects of water stress on growth and nitrogen-fixing activity of Trifolium repens. New Phytol. 72:117-126.
24. Farnsworth, R.B. and M.A. Clawson. 1972. Nitrogen fixation by Artemisia ludoviciana determined by acetylene-ethylene gas assay. Agron. Abst. Amer. Soc. Agron. 96.

25. Fessenden, R.J., R. Knowles, and R. Brouzes. 1973. Acetylene-ethylene assay studies on excised root nodules of Myrica asplenifolia L. Soil Sci. Soc. Am. Proc. 37:893-898.
26. Furman, T.E. 1959. The structure of the root nodules of Ceanothus sanguineus and Ceanothus velutinus, with special reference to the endophyte. Amer. J. Bot. 46:698-703.
27. Gardner, I.C. 1958. Nitrogen fixation in Elaeagnus root nodules. Nature 181:717-718.
28. Gardner, I.C. and G. Bond. 1957. Observations on the root nodules of Shepherdia. Can. J. Bot. 35:305-314.
29. Gibson, A.H. 1965. Physical environment and symbiotic nitrogen fixation. II. Root temperature effects on the relative nitrogen assimilation rate. Aust. J. Biol. Sci. 18:295-310.
30. Hardy, R.W.F., R.C. Burns, and R.D. Holsten. 1973. Applications of the acetylene-ethylene assay for measurement of nitrogen fixation. Soil Biol. Biochem. 5:47-81.
31. Hardy, R.W.F., R.D. Holsten, E.K. Jackson, and R.C. Burns. 1968. The acetylene-ethylene assay for N_2 fixation: laboratory and field evaluation. Plt. Physiol. 43:1185-1207.
32. Hardy, R.W.F. and E. Knight, Jr. 1967. ATP-dependent reduction of azide and HCN by N_2 -fixing enzymes of Azotobacter vinelandii and Clostridium pasteurianum. Biochim. Biophys. Acta 139:69-90.
33. Hawker, L.E. and J. Fraymouth. 1951. A re-investigation of the root-nodules of species of Elaeagnus, Hippophae, Alnus and Myrica with special reference to the morphology and life histories of the causative organisms. J. Gen. Microbiol. 5:369-386.
34. Hiltner, L. 1896. Über die bedeutung der wurzelknollen von Alnus glutinosa für die stickstoffernährung dieser pflanze. Landw. Vers. Sta. 46:153-161.
35. Hoeppel, R.E. and A.G. Wollum, II. 1971. Histological studies of ectomycorrhizae and root nodules from Cercocarpus montanus and Cercocarpus paucidentatus. Can. J. Bot. 49:1315-1318.

36. Hwang, J.C., C.H. Chen, and R.H. Burris. 1973. Inhibition of nitrogenase-catalyzed reductions. *Biochim. Biophys. Acta* 292:256-270.
37. Kohnke, H. 1941. The black alder as a pioneer tree on sand dunes and eroded land. *J. Forestry* 39:333-334.
38. Lalonde, M. and J.-A. Fortin. 1972. Formation de nodules racinaires axéniques chez Alnus crispa var. mollis. *Can. J. Bot.* 50:2597-2600.
39. Lawrence, D.B. 1958. Glaciers and vegetation in southeastern Alaska. *Amer. Scientist* 46:89-122.
40. Lawrie, A.C. and C.T. Wheeler. 1973. The supply of photosynthetic assimilates to nodules of Pisum sativum L. in relation to the fixation of nitrogen. *New Phytol.* 72:1341-1348.
41. Lehninger, A.L. 1970. *Biochemistry*. Worth Pub., Inc., N.Y.
42. Linvill, D.E. 1972. Microclimatic radiation and the growth rate of corn. Ph.D. Thesis, Purdue Univ., West Lafayette, Indiana.
43. Mague, T.H. and R.H. Burris. 1972. Reduction of acetylene and nitrogen by field-grown soybeans. *New Phytol.* 71:275-286.
44. McKee, H.S. 1962. *Nitrogen Metabolism in Plants*. Clarendon Press, Oxford.
45. McNiel, R.E. and P.L. Carpenter. 1972. Nitrogen availability to woody plant species as influenced by nitrogen fixation. Interim Report, Joint Highway Research Project, Purdue Univ., Lafayette, Indiana. July 1972-No. 13.
46. McNiel, R.E. and P.L. Carpenter. 1974. Nitrogen fixation by woody plant species as measured by the acetylene reduction assay. *HortScience* 9:381-382.
47. McNiel, R.E. and P.L. Carpenter. 1976. Effect of temperature on C_2H_2 -reducing activity associated with root nodules on woody trees and shrubs. *J. Amer. Soc. Hort. Sci.* (In preparation).

48. McNiel, R.E. and P.L. Carpenter. 1976. Factors influencing the reduction of acetylene by excised root nodules from Elaeagnus umbellata 'Cardinal'. Soil Sci. Soc. Amer. Proc. (In preparation).
49. Meyeh, J. 1829. Ueber das herauswachsen parasitischer gewächse aus den wurzeln anderer pflanzen. Flora (Jena) 12:49-63.
50. Morris, M., D.E. Eveleigh, S.C. Riggs, and W.N. Tiffney, Jr. 1974. Nitrogen fixation in the bayberry (Myrica pensylvanica) and its role in coastal succession. Amer. J. Bot. 61:867-870.
51. Pate, J.S. 1961. Temperature characteristics of bacterial variation in legume symbiosis. Nature 192: 637-639.
52. Philips, D.A. and J.G. Torrey. 1972. Studies on cytokinin production by Rhizobium. Plt. Physiol. 49:11-15.
53. Postgate, J.R. 1974. New advances and future potential in biological nitrogen fixation. J. Appl. Bact. 37:185-202.
54. Quispel, A. 1954. Symbiotic nitrogen-fixation in non-leguminous plants. I. Preliminary experiments on the root-nodule symbiosis of Alnus glutinosa. Acta Bot. Neerl. 3:495-511.
55. Rodriguez-Barrueco, C. 1966. Fixation of nitrogen in root nodules of Alnus jorullensis H.B. & K. Phytion 23:103-110.
56. Rodriguez-Barrueco, C. 1968. The occurrence of nitrogen-fixing root nodules on non-leguminous plants. Bot. J. Linn. Soc. 62:77-84.
57. Rodriguez-Barrueco, C. and F. Bermudez de Castro. 1973. Cytokinin-induced pseudonodules on Alnus glutinosa. Physiol. Plant. 29:277-280.
58. Rogers, R.D. and A.G. Wollum, II. 1974. Virulence of the Alnus endophyte after in vitro cultivation. Soil Sci. Soc. Amer. Proc. 38:756-759.
59. Schollhorn, R. and R.H. Burris. 1966. Study of intermediates in nitrogen fixation. Fedn. Proc. Fedn. Am. Soc. Exp. Biol. 25:710.

60. Schollhorn, R. and R.H. Burris. 1967. Acetylene as a competitive inhibitor of N_2 fixation. Proc. Nat'l Acad. Sci. U.S.A. 58:213-216.
61. Silver, W.S. 1964. Root Nodule Symbiosis. Endophyte of Myrica cerifera L. J. Bacteriol. 87:416-421.
62. Silver, W.S. 1969. Biology and ecology of nitrogen fixation by symbiotic associations of non-leguminous plants. Proc. Roy. Soc. B. 172:389-400.
63. Silver, W.S. and T. Mague. 1970. Assessment of nitrogen fixation in terrestrial environments in field conditions. Nature 227:378-379.
64. Sloger, C. 1968. Nitrogen fixation by tissues of leguminous and nonleguminous plants. Ph.D. Thesis. Univ. Florida, Gainesville, Florida.
65. Snedecor, G.W. and W.G. Cochran. 1967. Statistical Methods. Iowa State Univ. Press, Ames, Iowa.
66. Spratt, E.R. 1912. The morphology of the root tubercles of Alnus and Elaeagnus, and the polymorphism of the organism causing their formation. Ann. Bot. 26:119-128.
67. Sprent, J.I. 1972. The effects of water stress on nitrogen fixing root nodules. IV. Effects on whole plants of Vicia faba and Glycine max. New Phytol. 71:603-611.
68. Sprent, J.I. 1973. Growth and nitrogen fixation in Lupinus arboreus as affected by shading and water supply. New Phytol. 72:1005-1022.
69. Stewart, W.D.P. 1966. Nitrogen fixation in plants. Univ. London, Athlone Press.
70. Stewart, W.D.P., G.P. Fitzgerald, and R.H. Burris. 1967. In situ studies on N_2 fixation using the acetylene reduction technique. Proc. Nat'l Acad. Sci. 58:2071-2078.
71. Tarrant, R.E. and J.M. Trappe. 1971. The role of Alnus in improving the forest environment. Plant Soil Spec. Vol.:335-348.

72. Uemura, S. 1964. Isolation and properties of micro-organisms from root nodules of non-leguminous plants. Bull. Govt. For. Expt. Sta. (Tokyo) 167:59-91.
73. Virtanen, A.I. and J.K. Miettinen. 1963. Biological nitrogen fixation. In: Plant Physiology. F.C. Steward, ed., Academic Press, N.Y. 3:539-668.
74. Virtanen, A.I., T. Moisiö, R.A. Allison, and R.H. Burris. 1954. Fixation of molecular nitrogen by excised nodules of the Alder. Acta Chem. Scand. 8:1730-1731.
75. Vlamis, J., A.M. Schultz, and H.H. Biswell. 1958. Nitrogen-fixation by deerbrush. Cal. Agr. Expt. Sta. Cal. Agr. 12(1):11.
76. Vlamis, J., A.M. Schultz, and H.H. Biswell. 1964. Nitrogen-fixation by root nodules of western mountain mahogany. J. Range Management 17:73-74.
77. Wagle, R.F. and J. Vlamis. 1961. Nutrient deficiencies in two bitterbrush soils. Ecology 42:745-752.
78. Waughman, G.J. 1972. The effect of varying oxygen tension, temperature and sample size on acetylene reduction by nodules of Alnus and Hippophae. Plant Soil 37:521-528.
79. Wheeler, C.T. 1969. The diurnal fluctuation in nitrogen fixation in the nodules of Alnus glutinosa and Myrica gale. New Phytol. 68:675-682.
80. Wheeler, C.T. 1971. The causation of the diurnal changes in nitrogen fixation in the nodules of Alnus glutinosa. New Phytol. 70:487-495.
81. Wheeler, C.T. and B.G. Bowes. 1974. Effects of light and darkness on nitrogen fixation by root nodules of Alnus glutinosa in relation to their cytology. Z. Pflanzenphysiol. Bd. 71:71-75.
82. Wollum, A.G. II and C.T. Youngberg. 1964. The influence of nitrogen fixation by nonleguminous woody plants on the growth of pine seedlings. J. Forestry. 62:316-321.
83. Wollum, A.G. II, C.T. Youngberg, and C.M. Gilmour. 1966. Characterization of a Streptomyces sp. isolated from the root nodules of Ceanothus velutinus Dougl. Soil Sci. Soc. Amer. Proc. 30:463-467.

84. Ziegler, H. and R. Huser. 1963. Fixation of atmospheric nitrogen by root nodules of Comptonia peregrina. Nature 199:508.

APPENDIX

APPENDIX

Measurement of Acetylene-Ethylene

Ethylene was measured with a Varian 1200 gas chromatograph.

Specifications:

detector: flame ionization detector, temperature 160 C

injector: Temperature 110 C

column: length 150 cm x 1/8" O.D. stainless steel,
packed with 1.8 g Porapak R, 80/100 mesh (supplied by McFaul Company, Cincinnati, Ohio),
temperature 40 C

carrier gas: nitrogen gas

gasflow: 40 ml/min

retention time: C_2H_4 = 49 sec, C_2H_2 = 68 sec

Each gas sample injected contained 1 ml of gas.

COVER DESIGN BY ALDO GIORGINI